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Induction of Tumors by a Virus-Like Agent(s)* Released by Tissue Culture. (24227)

EDWIN A. MIRAND, DAVID T. MOUNT, GEORGE E. MOORE, JAMES T. GRACE, JR. AND JOSEPH E. SOKAL

Roswell Park Memorial Institute, Buffalo, N. Y.

Borrel first held the concept that a virus might be a causative agent in cancer. However, viruses resemble bacteria, protozoa and fungi in causing lesions which range from suppurative or necrotizing lesions to granulomatous or proliferative ones depending on severity and type of injury. Neoplasia may represent an extreme example of the proliferative type of reaction or develop as a result of hyperplasia. Rous(1) reported transmission of a sarcoma in a Plymouth Rock hen by cell-free filtrates. Our knowledge of cell-free filtrates and cell particulates as they effect neoplastic processes has increased enormously since this period(2-26 and Table I). Gross has reported that filtered cell-free leukemia extracts of AK mice when inoculated into newborn C_3H mice can induce leukemia, parotid tumors and subcutaneous sarcomas (2,3,4). Recently Gross(5) reported the high susceptibility of 1- to 14-day-old C_3H mice to "passage A" leukemia filtrates inducing leukemia but not parotid tumors or sarcomas. Stewart(6) produced neoplasms of the parotid gland and adrenal gland in mice inoculated with cell-free extracts or filtrates of leukemic mouse tissues. Drs. Stewart *et al.* and Eddy *et al.*(7,8) reported a tissue culture method to increase the tumor-producing capacity of leukemic extracts or parotid gland tumors by incubating them in tissue cultures of cells from monkey kidney or from mouse embryos. The increase in tumor-producing capacity was shown by production of a wider variety of tumors and a pronounced decrease in the tumor latent period. The tumor-producing effect from these fluids of tissue culture preparations was *neither strain nor species specific*(7,8,9).

This report confirms and extends, by using inbred strains of mice, rats, and hamsters, the recent observations of Stewart and Eddy relative to induction of multiple types of cancers in hamsters(9), mice(7,8), and rats(10) with a virus-like agent derived from a mouse tumor propagated in mouse embryo tissue culture.

Material and method. Tissue cultures were prepared as reported by Eddy *et al.*(9). Swiss embryos of the ICR-Ha strain approximately in the 12th day of gestation were used for our mouse embryo tissue culture preparations.

Sources of tumor-producing material. Tissue culture fluids containing the virus-like agent were obtained from Dr. Stewart in November, 1957. These were unfiltered fluid #3919, 3791, and D-3919 (lyophilized). Our own tissue culture fluids (M-1-1 and M-1-2)

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TABLE I. Induction of Tumors by Cell-Free Extracts in Mammals.

Investigator	Source of material	Responsive recipient	Resulting lesions
Gross (1951-1958)	AKR and C_{58} tissues	Newborn and young C_3H/Bi, C_{57} Bl	Lymphatic leukemia, few parotid tumors and associated neoplasms
Stewart and Eddy (1955-1958)	AK leukemia, Swiss salivary tumors and other tissues	Newborn mice C_3Hf x AKR F_1 Swiss Newborn and young hamsters Newborn rats " rabbits	Multiple types of neoplasms and/or hyperplasia
Schwartz *et al.* (1957-1958)	Swiss leukemia C_3H " AKR " Human "	Adult Swiss " C_3H " AKR " AKR	Only brain filtrate is effective in producing lymphatic leukemia
Friend (1957-1958)	Ehrlich neoplasm	Adult Swiss, DBA/2	Leukemic-like disease
Graffi *et al.* (1956-1958)	Ehrlich neoplasm	Young Agnes-Bluhm mice	Acute and chronic chloroleukemia
Mirand *et al.* (1958)	Swiss salivary tumors	Newborn DBA/1, AKR/Sn 57 Black/Ha, Swiss/ICR-Ha AKR x Swiss F_1 Newborn and young hamsters Newborn rats	Multiple types of neoplasms and/or hyperplasia

containing the tumor inducing agent(s) were obtained by inoculating Swiss embryo (ICR-Ha) tissue cultures with minces of a parotid tumor from a Swiss mouse (NIH). Culture fluids to which *E. coli* suspensions were added were filtered through HA grade millipore filters and fluids were tested before and after filtering for *E. coli*. Newborn mice were inoculated with filtered and unfiltered tissue fluids from these tissue cultures.

Control tissue cultures. Control tissue culture fluids were also obtained from Dr. Stewart. Our own control tissue culture fluids were obtained from Swiss embryo (ICR-Ha) tissue cultures inoculated with normal Swiss parotid tissue.

Hamsters. Hamsters (*Cricetus anuratus*) used in this study were obtained from the Nat. Inst. of Health and Blue Spruce Farms. Cannibalism of newborn and young hamsters by their mothers was a serious problem. The hamsters, at time of injection, varied in age from newborn to 2 weeks. The dose of tissue culture fluid administered to each animal was 0.1 ml by subcutaneous injection.

Mice. Mice used in this study were bred at the Institute. These were C_{57} Black/Ha, DBA/1, Swiss/ICR-Ha, AKR/Snell and AKR x Swiss F_1 hybrids. Some Swiss mice of the NIH strain obtained from Dr. Stewart were used. Newborn mice, less than 24 hours old, were injected subcutaneously with 0.1 ml of tissue culture fluid.

Rats. Rats of the Sprague-Dawley strain were obtained from Holtzman Co., Madison, Wisc. Newborn rats less than 24 hours old were injected subcutaneously with 0.1 ml of tissue culture fluid.

Results. Table II presents a summary of types and sites of tumors induced in animals by different tissue culture fluids of Dr. Stewart and of our own (M-1-1 and M-1-2).

Table III presents induction of tumors in animals injected subcutaneously when less than 24 hours old with 0.1 ml of unfiltered tissue culture fluid received from Dr. Stewart. The average tumor latent periods for hamsters, mice and rats were 29, 75, and 180 days, respectively. Three out of the 6 AKR/Sn mice receiving tissue culture fluid 3791 developed both leukemias and parotid tumors in 2½ months, while in the others only parotid tumors were observed. It is significant that C_{57} Bl/Ha mice, a "low tumor" strain, developed parotid tumors with tissue culture fluid, #3919 and D-3919. DBA/1 mice, high mammary tumor strain, developed salivary gland tumors and osteogenic sarcomas.

TABLE II. Induction of Tumors in Animals by Different Tissue Culture Fluids.

Animal	Type	Site	Avg latent period (days)
Hamster	Sarcoma	Heart, kidney, blood vessel, stomach, mediastinum, lung, subcut.	29
	Vascular*	Lung, liver	
Mouse	Leukemia	Thymus, lymph node, liver	75
	Osteogenic sarcoma	Rib, vertebra, shoulder	
	Sarcoma	Lung, kidney, subcut., heart	
	Mixed–carcinoma (pleomorphic)	Salivary glands (parotid, submaxillary, sublingual)	
Rat	Squamous cell carcinoma	Lip	180
	Sarcoma	Caecum, kidney,† lung	

* Duran-Reynals also observed vascular lesions in chickens(26).
† Kidney tumor in its F_3 transplantable generation; 100% takes.

In Table IV are data from our own tissue culture fluids prepared by the technic of Eddy and Stewart(9). It can be observed that tissue culture fluids of the first week from first passage (M-1-1) displayed tumor-inducing ability in hamsters injected shortly after birth. Moreover, tissue culture fluids of the first and second week from second passage (M-1-2) displayed tumor inducing ability in newborn mice and newborn hamsters. The average latent periods were similar to those in Table II.

Recently 2 DBA/1 mice, one Swiss/ICR-Ha mouse, and one hamster injected when newborn with control tissue culture fluid (line 1) developed parotid tumors and in the hamster multiple tumors (heart, kidney, lung, and liver). Although rigid precautions in technic were observed, the possibility should be considered that the virus-like agent(s) contaminated our control tissue culture fluid or that the normal Swiss mouse embryo harbors in low concentration a virus-like agent(s) and that embryo cells *in vitro* increase its concentration. The fact that only one batch of control material (line 1) was oncogenic adds credence particularly to the first possibility.

Attempts to transplant some of these induced tumors occurring in inbred mouse strains, particularly parotid, into the strain of origin have been successful, when newborn and pre-conditioned irradiated hosts were used. This result should be considered in interpreting neoplastic nature of the growths observed.

Discussion. Stewart and Eddy have re-

TABLE III. Induction of Tumors in Newborn Animals by a Virus-Like Agent(s) in Tissue Culture Fluids Obtained from Dr. S. Stewart.

Tissue culture strain	Animal	Injected	Effective survival	No. animals died with tumors	Surviving without tumors
3919	Hamsters	115	5	5	0
	Swiss/ICR-Ha	31	20	8	10 (2)
	C_{57} Black/Ha	8	3	1	2
	DBA/1	5	3	3	0
	Rat	91	32	8	12 (12)
3791	Swiss/ICR-Ha	50	24	2	17 (5)
	C_{57} Black/Ha	10	8	1	6 (1)
	DBA/1	10	4	0	3 (1)
	AKR/Sn	7	6	6	0
	AKR x Swiss F_1	9	9	2	7
D-3919	DBA/1	8	0	0	0
	C_{57} Black/Ha	6	4	2	1 (1)
	Rat	29	11	1	8 (2)

Control fluids inj. into control animals showed to date no evidence of tumor development.
( ) Animals died without tumors.

TABLE IV. Induction of Tumors in Newborn Animals with Virus-Like Agents in Tissue Culture Fluids.*

Tissue culture fluid (Line M-1)	Animal	Injected	Effective survival	No. animals died with tumors	Surviving without tumors
(A) First passage					
M-1-1 2nd wk (unfiltered)	Hamster	11	5	4	0 (1)
	Swiss/ICR-Ha	13	10	0	7 (3)
Idem (filtered)	Hamster	10	3	2	0 (1)
	Swiss/ICR-Ha	7	0	0	0
(B) Second passage					
M-1-2 1st wk (unfiltered)	Swiss/ICR-Ha	11	8	0	6 (2)
	C_{57} Black/Ha	4	2	1	1
Idem (filtered)	Swiss/ICR-Ha	16	12	6	4 (2)
	C_{57} Black/Ha	7	5	0	5
M-1-2 2nd wk (unfiltered)	DBA/1	6	2	2†	0
	Swiss/ICR-Ha	11	8	4	4
Idem (filtered)	Hamsters‡	10	3	3	0
	Swiss/ICR-Ha	15	0	0	0
	DBA/1	3	2	1	0 (1)

Control tissue culture fluids of first and second wk did not show to date evidence of tumor development.

( ) Animals died without tumors.

* Mouse embryo tissue culture inoculated with minces from Swiss mouse parotid tumor.

† Intranuclear inclusion bodies in kidney cells.

‡ 14-day-old hamsters.

ferred to the tumor inducing agent(s) as the SE-polyoma virus. Virological data reported by Eddy(10) and Stewart(11) suggest the existence of one virus rather than a family of viruses. Further study of this facet is indicated. It is surprising, indeed, to observe that this virus-like agent(s) is not strain or species specific. The variety of tumors that one sees in the injected animals appears to be determined by host susceptibility to the virus-like agent(s). The same response is frequently seen with carcinogens. Although the hamster appears to be very susceptible to the agent as judged by a very short latent period, the mouse comes down with a greater variety of tumors. Whether these tumors that arise are multiple primaries or metastatic tumors is still not definitely established. However, it appears that they are not metastatic in nature.

Among the responsive recipients, newborn mice and rats, as well as newborn and 2-week-old hamsters are susceptible to inoculation of the SE-polyoma virus or virus-like agent(s). In studies on chicken leukosis(15,16,17,18) and on Rous Sarcoma(19) and other related studies (Table I) very young and/or young recipients are also more sensitive to cell-free extracts. Whether there is an optimum age factor involved in the susceptibility of recipients to the SE-polyoma virus remains to be more fully examined. The data from the laboratory of Stewart and Eddy seem to support the tentative conclusion that there is an optimum age factor. Generally there seems to be decreased susceptibility and increased latent period with advancing age. This, of course, agrees quite well with the findings of others who have induced tumors with cell-free filtrates. Immunological immaturity of the newborn, genetic features of the host, metabolic state of the host cells and presence of other viruses may all play a role in vulnerability of the host tissues to the SE-polyoma virus.

It has been reported(11) that when breeding units of mice are maintained in the same room with mice that have been injected with tissue culture fluids the newborn mice from such a colony remain resistant to the virus-like agent(s) in the tissue culture fluid. This is suggestive of immunity after exposure. It is possible that passive transfer of antibodies from the mothers may have conferred some

degree of immunity to the newborns. This problem is under investigation in our laboratory.

The intracellular mechanisms underlying viral oncogenesis remain to be elucidated. Whether tumor formation results from alteration of cells directly by viral parasitism of specific substances, such as RNA, or is mediated more indirectly through interference with homeostatic growth regulation in the host, or both, is still unanswered.

Stasney *et al.*(24) and Paschkis *et al.*(25) induced lymphosarcomas and hepatomas in rats with chromatin and to a much lesser extent with mitochondria fractions from both tumors. Even though tissue culture fluids still possessed tumor inducing properties upon being passed through filters as small as a pore size of 77 mμ(10), the possibility remains that the tumors might be related to cellular particulates rather than a virus. However, it remains to be shown whether the hypothetical activity of such cell particulates and infectious agents is experimentally separable.

Summary. (1) The observations reported by Stewart and Eddy have been confirmed and extended by using inbred strains of mice, rats, and hamsters treated with tissue culture fluids prepared by Stewart and Eddy and at this laboratory. (2) Injection of such tissue culture fluids into newborn and young animals produced multiple types of tumors in mice of DBA/1, Swiss/ICR-Ha, C_{57} Black/Ha, AKR x Swiss F_1, and AKR/Sn strains as well as in hamsters and rats. (3) Although most control tissue culture fluids possessed no tumor inducing ability, in one instance a control tissue culture fluid (line 1) did produce parotid tumors in 2 DBA/mice, 1 Swiss/ICR-Ha mouse, and multiple tumors in 1 hamster. (4) Transplantation of some of these induced tumors occurring in inbred mouse strains has been successful into the strain of origin if newborn and preconditioned irradiated hosts are used.

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Development of Susceptibility to Seizures in Young Animals III. Brain Water, Electrolyte and Acid-Base Metabolism.* (24228)

J. Gordon Millichap,† Mana Balter and Patricio Hernandez
Departments of Pediatrics and Pharmacology, Albert Einstein College of Medicine, Yeshiva University, and the Bronx Municipal Hospital Center, New York City

In a previous study concerning development of susceptibility to experimental seizures in young animals, newborn rats were found to be much less susceptible than older rats(1). From birth to 10 days of age, the response to an electroshock stimulus of 50 m. amps. consisted of hyperkinetic behavior and opisthotonic spasms, and neither clonic nor tonic convulsions could be induced. Between 10 and 20 days, a rapid increase in seizure susceptibility was observed and the severity of the seizure pattern progressed from minor tremors to generalized clonic convulsions. Susceptibility was greatest at 25 to 35 days of age, when a maximal seizure response was obtained with tonic extension of all limbs. In older and adult rats, stronger electroshock stimuli are required for induction of major seizures and reduction in susceptibility occurs with increasing age(2). In the guinea pig, unlike the rat, there was little change in the electroshock seizure response from birth to 35 days of age. The clonic response predominated and a maximal seizure with a hindlimb tonic extensor component could not be induced.

In order to elucidate possible mechanisms in the seizure process, the changes observed in the seizure threshold with age have been studied in relation to development of carbonic anhydrase activity in the brain. In the rat, a sharp increase in brain carbonic anhydrase occurs at 10 to 20 days of age whereas in the guinea pig, little change in enzyme activity is observed during the first month of life. The degree of maturation of the newborn guinea pig corresponds with that of the 15- to 20-day-old rat, both with regard to seizure susceptibility and level of brain carbonic anhydrase activity. By developmental studies and by the use of acetazolamide, an anticonvulsant which inhibits carbonic anhydrase, a direct relation has been demonstrated between activity of the enzyme in the brain and susceptibility to experimental seizures in young animals of different species(3). The specific catalytic activity of brain carbonic anhydrase appears to be essential for propagation of the seizure discharge.

Studies in adult rats have shown that the anticonvulsant effect of acetazolamide is associated with an increase in the cellular total carbon dioxide concentration in the brain and with changes in the distribution of brain electrolytes.‡ Woodbury *et al.* have drawn attention to the anticonvulsant properties of carbon dioxide and the effects of brain electrolyte alterations on the seizure threshold (4). In the present developmental study, susceptibility to experimental seizures and concentration of brain carbonic anhydrase have been correlated with brain water and electrolyte metabolism in rats and guinea pigs and with the carbon dioxide content of the brain in rats.

Methods. Newborn, one-month-old and adult rats of the Holtzman and Sprague-Dawley strains and newborn guinea pigs were used as experimental animals. The percentage of animals which showed a maximal tonic extensor seizure in response to an electroshock stimulus of 50 m. amps. applied for 0.2 sec. was used as a measure of seizure susceptibility. An average of 25 rats in each age group and 12 guinea pigs were employed.

Water and electrolyte analyses. Animals of the same age as those tested for seizure susceptibility were used for estimations of water and electrolyte concentrations of plasma and brain tissue. Animals were killed by de-

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† Present address: Massachusetts General Hosp., Boston.

‡ Woodbury, D. M.: Personal communication.

capitation, blood was collected in tubes containing heparin, and samples of the cerebral cortex and subcortical white matter were transferred to tared beakers, after removal of macroscopic blood and surface vessels. In newborn rats, samples from one litter of 8 to 10 animals were pooled for each determination. In older and adult rats and in guinea pigs, samples from 2 to 4 animals were pooled. Plasma and brain tissue were dried at 105°C to a constant weight; water content was calculated from the difference between wet and dry weights. Nitric acid was added to the dried tissues which were digested by gentle heating; the resulting solutions were analyzed for sodium and potassium by means of a Process and Instruments flame-photometer with an internal lithium standard. Tissue chloride determinations were performed by the method of Van Slyke and Sendroy(5); plasma chloride determinations, by the method of Schales and Schales(6). In calculations of cellular concentrations of water, sodium and potassium, the chloride space was used as a measure of extracellular fluid volume in the brain(7). As a method for estimation of absolute values, the limitations of the chloride space are recognized but, as a measure of relative values and of directional changes in the volume of the fluid compartments of the brain with growth, objections to the method might be lessened. In order to make possible the calculations of standard errors of values for water and electrolyte concentrations for comparative purposes, an analysis of intracellular cations was made for each pooled sample rather than using the mean values calculated for each group of animals.

Tissue carbon dioxide determinations. The method of Danielson and Hastings(8) was used for estimation of the total carbon dioxide content of the cerebrum of rats. The brain was removed after decapitation and was transferred rapidly to the tube containing ferric fluoride and air-free sodium hydroxide. Estimations were performed on 0.5 to 1 g of tissue and the brains of 2 to 4 newborn rats were pooled. A total of 47 determinations were made in animals at 9 different ages between

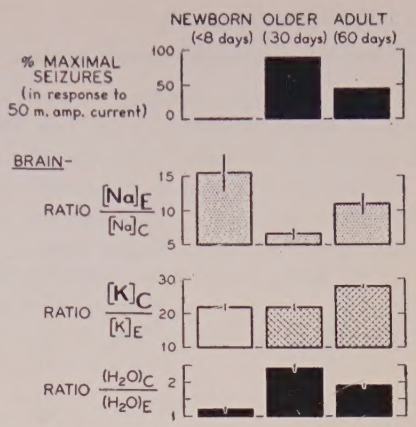

FIG. 1. Susceptibility to maximal electroshock seizures in newborn, older and adult rats in relation to distribution of brain water and electrolyte concentrations.

3 and 38 days. Values obtained in rats bred from the Holtzman strain were compared with those of the Sprague-Dawley strain. Since the consistency of the brain of older rats differed from that of the newborn, the values obtained with whole brain were compared with those for brain brei. A magnetic stirring rod was used to agitate the tissue and facilitate the evolution of the carbon dioxide. In addition to developmental studies, the brain carbon dioxide was estimated in 7 rats of the Sprague-Dawley strain aged 24 days, which were killed 2 hours after the subcutaneous injection of acetazolamide, in a dose of 1 g/kg/body weight. Control animals were examined after injection of an equal volume of isotonic saline. The collection of blood under anaerobic conditions was impractical in newborn rats and a comparison of intracellular pH and carbon dioxide concentration in the brain of newborn and older rats was not possible.

Results. Susceptibility to seizures. Fig. 1 shows degree of susceptibility to electroshock seizures in rats tested with a current of 50 m. amps. for 0.2 sec. Rats aged 30 days were most susceptible; a maximal response with tonic extension of the hind-lambs occurred in 90% of animals. Newborn rats between 4 and 7 days of age failed to respond maximally and seizure activity consisted of hyperkinetic behavior. In adult rats aged 60 days (55 to 65) maximal seizures were obtained

TABLE I. Concentrations of Water and Electrolytes in Brain and Plasma of Rats and Guinea Pigs.

		Cerebrum					Plasma			
Age of animal	No. of samples	H_2O, %	Na meq/km/wet tissue	K	Cl	No. of samples	H_2O, %	Na meq/kg/plasma	K	Cl
Newborn rat (4-7 days)	27[a]	87.61 ± .16*	61.45 ± .58	82.43 ± .56	43.02 ± .75	18	94.89 ± .32	135.9 ± 1.7	7.68 ± .21	98.33 ± 1.54
P_1 values		<.001	<.001	<.001	<.001		<.01	N.S.	N.S.	<.001
Older rat (28-32 days)	11[b]	81.30 ± .71	47.9 ± 1.03	99.2 ± 1.89	30.8 ± .68	11	93.4 ± .28	139 ± 1.33	7.4 ± .20	114.5 ± 2.22
P_2 values		<.01	N.S.	<.02	N.S.		<.01	<.05	N.S.	<.001
Adult rat (55-65 days)	5[c]	78.3 ± .40	48 ± .43	104.5 ± .50	31.6 ± .89	5	92.1 ± .18	143.7 ± 1.43	6.97 ± .06	101.5 ± .55
Newborn guinea pig (3-5 days)	6[d]	81.4 ± .20	52.7 ± .64	97.2 ± .72	37.5 ± .66	6	93.2 ± .37	136.1 ± 2.98	6.74 ± .08	109.4 ± 1.59
P_3 values		<.001	<.001	<.001	<.001		<.01	N.S.	<.01	<.05

[a,b,c,d]; each sample represents pooled specimens of brain or plasma obtained from 10, 4, 3, and 2 animals respectively.

P_1, P_2; tests of significance for differences between newborn and older, and older and adult rats, respectively.

P_3; tests of significance for difference between newborn rats and newborn guinea pigs.

* Mean ± stand. error.

only in 45% of the animals tested; a generalized clonic seizure was observed in the remainder. In the newborn guinea pig, seizures in response to a 50 m. amp. electroshock stimulus were unlike those in the newborn rat and corresponded more closely to responses obtained in the rat aged 15 to 20 days and the adult rat. Generalized clonic movements were preceded by tonic extension of the forelimbs but a maximal seizure with extension of the hind-limbs was observed rarely.

Water and electrolyte analyses. Table I shows concentrations of water and electrolytes in brain and plasma of newborn, 1-month-old and adult rats and of newborn guinea pigs. Table II shows the intracellular $()_C$ and extracellular $()_E$ concentrations of water, sodium and potassium in the brain. In Fig. 1, ratios of cellular to extracellular brain water and electrolyte concentrations are shown in relation to seizure susceptibility in rats. In newborn compared with older and adult rats, the potassium concentration in whole brain is 18% lower and the sodium and chloride, 27% and 40% higher, respectively. The water concentration of the newborn compared with adult brain is 10% higher but the ratio of cellular to extracellular brain water is approximately one-half that of the older animals. Ratios of cellular to extracellular potassium and of extracellular to cellular sodium in the brain of 30-day-old rats are lower than in adults. The highest ratio of extracellular to cellular sodium in the brain is found in newborn rats. In the newborn guinea pig, the concentration and distribution of water and electrolytes in the brain are significantly different from the values obtained in the newborn rat. The newborn guinea pig corresponds more closely to the older and adult rat, both with regard to seizure susceptibility and brain water and electrolyte metabolism.

Brain carbon dioxide content. Fig. 2 shows total carbon dioxide content of the brain in

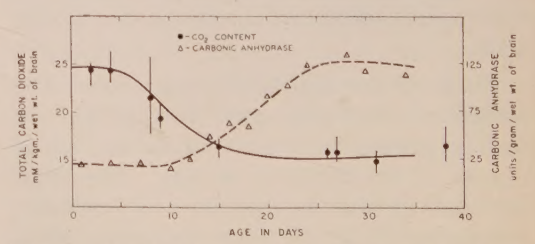

FIG. 2. Total carbon dioxide content of brain of rats 1 to 38 days of age in relation to brain carbonic anhydrase activity. Each point and vertical line represents respectively the mean and range of separate estimations on an avg of 5 specimens. Triangles are mean values of separate estimations on each of 2 to 4 animals.

TABLE II. Distributions of Water and Electrolytes in Brain of Rats and Guinea Pigs.

Age of animal	Water			Sodium			Potassium		
	Intracellular	Extracellular	Ratio†	Extracellular	Intracellular	Ratio†	Intracellular	Extracellular	Ratio†
	% whole tissue			meq/kg water			meq/kg water		
Newborn rat (4-7 days)	47.30 ± 1.36*	40.20 ± 1.48	1.23 ± .09	137.5 ± 1.94	15.15 ± 3.28	15.45 ± 2.73	169.0 ± 3.53	7.76 ± .23	22.0 ± .62
P_1 values	=.001	<.001	<.001	<.05	N.S.	<.01	N.S.	N.S.	—
Older rat (23-32 days)	57.20 ± 1.44	24.20 ± .93	2.42 ± .13	143.0 ± 1.43	22.1 ± 2.27	6.46 ± .67	165.3 ± 6.37	7.5 ± .22	22.0 ±1.04
P_2 values	<.01	<.01	<.01	<.01	<.01	=.02	<.001	<.02	<.001
Adult rat (55-65 days)	50.80 ± .77	27.60 ± .65	1.85 ± .07	149.8 ± 1.67	13.2 ± 1.27	10.90 ± 1.56	202.1 ± 2.73	7.25 ± .06	27.9 ± .51
Newborn g. pig (3-5 days)	50.7 ± .39	30.7 ± .32	1.65 ± .03	142.1 ± 3.37	18.1 ± 2.20	8.92 ± 1.82	187.2 ± 1.27	6.91 ± .03	27.1 ± .24
P_3 values	<.05	<.001	<.001	N.S.	N.S.	=.05	<.001	<.001	<.001

* Mean ± stand. error. † Mean of ratios obtained from individual samples.
P_1, P_2; tests of significance for differences between newborn and older, and older and adult rats respectively.
P_3; tests of significance for difference between newborn rats and newborn guinea pigs.

rats 2 to 38 days of age, bred from the Holtzman strain. When compared with carbonic anhydrase, a relatively high level of carbon dioxide in the newborn brain is associated with a low enzyme concentration. In 26-day-old rats which have a high level of brain enzyme activity, brain carbon dioxide content is 38% lower than in newborn rats ($P<.001$). When compared with those observed in rats of the Holtzman strain, the values obtained in 6 Sprague-Dawley rats were equal in the 4-day-old but higher in 26-day-old animals. In this strain, a difference of 20% in the brain carbon dioxide content of newborn and older rats was observed ($P<.001$). In an initial study of 8 rats aged 38 days and 2 aged 2 days, the values obtained with brain brei were 1 mM/kg lower than those with whole brain. The difference was not significant ($P>0.1$), but all subsequent estimations were performed on whole brain.

Effect of acetazolamide on brain carbon dioxide. In 2 groups of 24-day-old rats treated with acetazolamide the brain carbon dioxide content was 20.3 and 23 mM/kg; values obtained in control animals were 18 and 19.2 mM/kg, respectively. Thus, inhibition of carbonic anhydrase by acetazolamide was associated with an increase in brain carbon dioxide of 12% ($P<.05$) in one group and 20% ($P< .001$) in a second group of rats.

Discussion. In the present series of investigations, susceptibility to maximal electroshock seizures in rats was greatest at one month of age. In newborn and adult rats, a low degree of susceptibility or high threshold to electroshock seizures was observed. The inability of the newborn rat to respond maximally with a tonic seizure pattern is in accordance with clinical observations and the infrequent occurrence of generalized tonic-clonic seizures in immature infants. We have observed in the newborn that the clinical pattern of a seizure consists principally of tremors and opisthotonic and myoclonic spasms. In infants and in adults, history of an inherited lowered threshold to seizures is relatively uncommon and seizures are associated usually with structural brain lesions. The age of greatest susceptibility is between 4 and

8 years, when incidence of those seizures unassociated with brain pathology is highest.

Changes in susceptibility to seizures with age have been correlated with the water, electrolyte and acid-base metabolism of the brain in animals of different species. Susceptibility to electroshock seizures of newborn, older and adult rats is related inversely to the ratio of extracellular to cellular sodium ($[Na]_E/[Na]_C$) and directly, to the ratio of cellular to extracellular water ($(H_2O)_C/(H_2O)_E$) in the brain. The large extracellular fluid space observed in the newborn may be explained by the relatively small nerve cells and the paucity of glia and cell processes at this age. However, the possible effect of an increased permeability of cell membranes to chloride ions cannot be excluded. In newborn and adult rats, susceptibility to electroshock seizures is related inversely to total carbon dioxide content of the brain. The reciprocal relation between carbon dioxide and carbonic anhydrase concentrations in the brain is seen in Fig. 2. In agreement with observations in adult rats,‡ inhibition of carbonic anhydrase by acetazolamide in 1-month-old animals is associated with an increase in carbon dioxide content of the brain. In the newborn guinea pig, an animal more mature than the rat at birth, degree of seizure susceptibility and concentrations of water and electrolytes in the brain resemble those of older and adult rats. In addition, brain carbonic anhydrase activity in the newborn guinea pig is greater than that in the newborn rat and corresponds more closely with the level of enzyme activity observed in older rats(1).

The relation between seizure susceptibility, carbonic anhydrase activity, and water, electrolyte and acid-base metabolism demonstrated in developmental studies is supported by observations in adult animals treated with acetazolamide. The anticonvulsant effect of acetazolamide is related directly to inhibition of brain carbonic anhydrase(9); it is associated with an accumulation of carbon dioxide in the brain which, in certain concentrations, is known to depress seizure activity. Acetazolamide decreases the permeability of brain cell membranes and the rate of uptake of radiosodium; anticonvulsant doses effect an increase in the ratio of extracellular to cellular brain sodium.‡ An increase in ratio of cellular to extracellular potassium ($[K]_C/[K]_E$) observed in adult rats treated with acetazolamide is not demonstrable in newborn rats which have a low level of brain carbonic anhydrase and a high threshold to seizures. However, when results obtained in 30-day-old and adult rats are compared, the correlation between seizure susceptibility and distribution of potassium is similar to that observed in acetazolamide-treated animals. The catalytic activity of carbonic anhydrase and the local acid-base changes produced are involved in transport and distribution of ions in brain tissue. Development of susceptibility to seizures in young animals is related to the maturation of carbonic anhydrase activity and to the effects of the enzyme on acid-base and water and electrolyte metabolism of the brain.

Summary. In newborn, 1-month-old and adult rats, susceptibility to maximal electroshock seizures has been correlated with distribution of water and electrolyte concentrations and total carbon dioxide content of the cerebrum. Seizure susceptibility is greatest at one month of age. It is related inversely to the ratio of extracellular to cellular sodium and directly to the ratio of cellular and extracellular brain water. The relation to distribution of potassium is inconstant. The newborn guinea pig, an animal more mature than the rat at birth, corresponds more closely to the older and adult rat, both with regard to seizure susceptibility and brain water and electrolyte metabolism. Failure to induce convulsions in the newborn rat is associated with a high level of total carbon dioxide in the brain. Development of seizure susceptibility in older rats is related to a rise in brain carbonic anhydrase activity and to a reciprocal fall in total carbon dioxide content of the brain.

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Hepatic Uptake and Biliary Excretion of Indocyanine Green in the Dog.* (24229)

HENRY O. WHEELER,† WILLIAM I. CRANSTON,‡ AND JAY I. MELTZER
(Introduced by Stanley E. Bradley)
Department of Medicine, Columbia University College of Physicians and Surgeons, and the Presbyterian Hospital in the City of New York

Indocyanine green is a water-soluble tricarbocyanine dye developed by Brooker and introduced by Fox *et al.*(1,2) for measurement of cardiac output by the indicator dilution technic. For this purpose its absorption spectrum and protein-binding characteristics (1) are ideally suited. The results of the present investigation indicate that the dye may also be of great value in study of hepatic function.

Methods. All studies were conducted on 2 trained unanesthetized female dogs weighing 18.9 and 22.0 kg. Several months previously each had undergone splenectomy, cholecystectomy, and preparation of a permanent duodenal fistula using the device described by Thomas(3), which permitted access to the common bile duct. The dogs remained in good health, and hepatic function, as judged by repeated measurements of sulfobromophthalein (BSP) transfer maximum(4), continued unimpaired. At the time of each study an olive-tipped ureteral catheter (5 or 6 Fr.) was inserted through the ampulla of Vater and advanced about 5 to 6 cm into the common bile duct. The dogs were then placed upright in a sling and bile was collected by gravity or, when it was particularly viscous, by gentle aspiration with a tuberculin syringe. Intravenous injections were given through a polyethylene catheter introduced through a large bore needle in a vein of the foreleg and advanced to the region of the right atrium.

Indocyanine green§ was made up to 250 mg% in distilled water for injections. In one study a continuous infusion of the dye was given. This was prepared by mixing 24 ml of 500 mg% indocyanine green with 51 ml of normal saline and 5 ml of dog plasma. (Indocyanine green is unstable on standing in aqueous solution, and the presence of plasma was found empirically to retard its breakdown.) Concentration of indocyanine green in plasma was measured in a Beckman DU spectrophotometer at 810 mμ after 11-fold dilution with normal saline. Concentration standards for this determination were also prepared in dog plasma. (Dilute aqueous standards are unsatisfactory because of marked instability.) Bile samples were diluted 25 to 100 times with water. One ml of diluted bile was then added to 1 ml of dog plasma and 9 ml of normal saline and the absorption measured at 810 mμ. Although the dye is quite stable in bile even when diluted, the addition of plasma, for reasons not apparent, was necessary for full color development. BSP concentrations

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† Markle Scholar in Medical Science.

‡ Rockefeller Travelling Fellow in Medicine.

§ Kindly supplied as "Cardio-Green" by Hynson, Westcott and Dunning, Inc., Baltimore, Md., through the courtesy of Dr. John H. Brewer, Director of Biological Research.

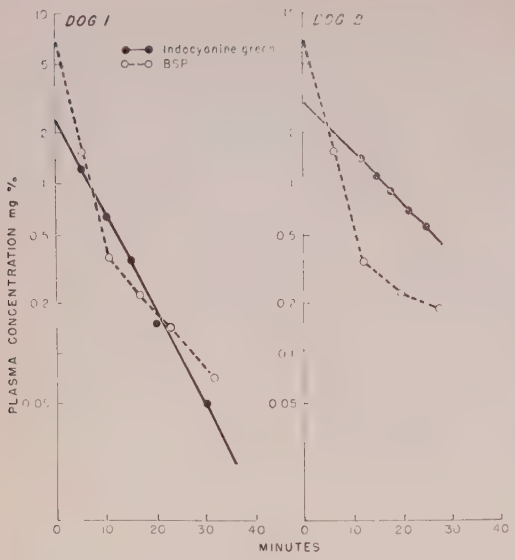

FIG. 1. Comparison of plasma concentrations of indocyanine green following administration of 20 mg intrav. with plasma concentrations of BSP following administration of 50 mg. Values are plotted semi-logarithmically against time after inj.

in plasma and bile were measured at 580 mμ after suitable dilution and addition of alkali. Measurement of the concentration of either dye was unaffected by the presence of the other.

Results. A. Disappearance from plasma: A single intravenous injection of 20 mg of indocyanine green was administered to each dog. During the succeeding 30 minutes plasma concentration fell exponentially with respect to time (Fig. 1). The half-times of the disappearance curves were 5.2 minutes and 10.5 minutes in dogs 1 and 2, respectively. Percentage disappearance rates(5) were 13.3% per min. and 6.6% per min. The curves were extrapolated back to zero time and this concentration value was divided into the administered dose to provide a rough estimate of the initial volume of distribution of injected material. This calculation yielded values of 830 and 706 ml compared to the measured plasma volumes (I^{131} labelled albumin) of 871 and 868 ml in these 2 dogs. The approximate agreement between volume of distribution and plasma volume strongly suggests that the initial distribution is limited to the vascular compartment. Following its introduction the dye must therefore be very rapidly and completely bound to plasma protein.

For comparison, an intravenous injection of 50 mg of BSP was given on another occasion to each of these dogs. Its initial disappearance rate was more rapid than that of indocyanine green (Fig. 1), but in contrast to the behavior of indocyanine green the disappearance of BSP did not follow an exponential course.

B. Biliary excretion. The recovery from bile of an intravenously administered dose of indocyanine green was close to 100% (Table I). No dye was detectable in the urine. Appreciable concentrations of dye were detectable in the bile for over 5 hours after injection in dog 1 and 4 hours in dog 2, long after its disappearance from the plasma (Fig. 2). These findings support the view that hepatic removal accounts for rapid disappearance of the dye from the plasma. Apparently indocyanine green, like some of the phthalein and flavine dyes(4,6,7,8,9), is rapidly stored in the liver and then gradually secreted into the biliary tree. The completeness with which it can be recovered in the bile by a colorimetric method suggests that it does not undergo major chemical alteration during this process. Moreover, in ascending paper chromatography with water saturated n-butanol-acetic acid the migration of indocyanine green recovered from several bile specimens was uniform and identical with that of the administered material. In contrast, BSP is excreted in several chromatographically distinct forms (10). The quantity of the latter dye identifiable in bile by colorimetric methods is rarely over 70 to 80% of an administered dose(6,7), and the measurement of S^{35} labelled BSP in the bile indicates that color is partially lost as a result of chemical alteration of this dye by the liver(11). In view of the behavior of in-

TABLE I. Recovery of Indocyanine Green in Bile.

Dog	Intrav. dose, mg	% recovered in bile
1	20	101.5
2	20	94.2
2	45	95.9
1	142	97.7
	Avg	97.3

docyanine green such modifications of the molecule do not appear to be essential either to storage or to transport.

C. Interference by indocyanine green with BSP uptake. A priming dose of BSP of approximately 10 mg/kg was administered intravenously and followed by a constant infusion of about 3.8 mg per min—a rate close to the BSP "Tm"(4) measured previously in these dogs and therefore designed to maintain a constant plasma level. After 30 minutes of infusion, control blood and bile specimens were obtained during three 10-minute intervals and then 45 mg of indocyanine green was administered to dog 2. During the first 10

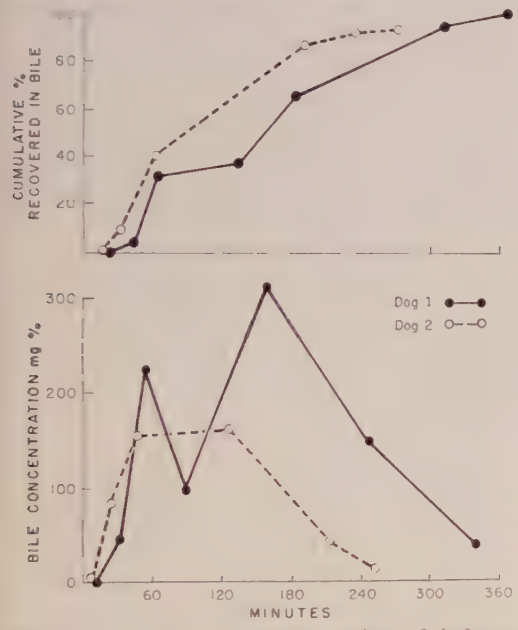

FIG. 2. Recovery and concentration of indocyanine green in bile following intrav. administration of 20 mg of the dye. Bile concentrations are plotted at mid-point of each collection period. Fluctuations in bile concentration in dog 1 were related to changes in bile flow.

minutes after this injection plasma concentration of BSP rose sharply from 3.6 to 5.1 mg % and then returned gradually to the control level over the next 100 minutes as the indocyanine green disappeared (Fig. 3). The biliary excretion rate of BSP showed a slight downward trend throughout the experiment and it was difficult to be sure whether this was modified during the period of maximum indocyanine green excretion. The ultimate

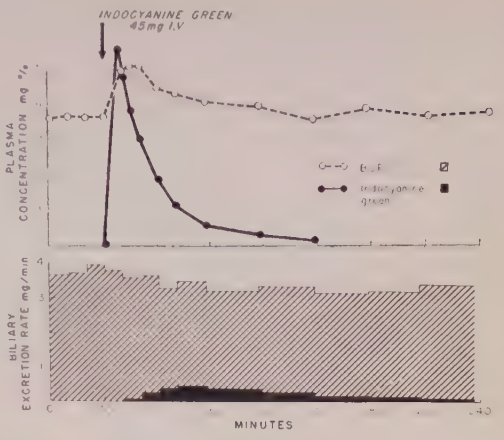

FIG. 3. Effect of a single intrav. inj. of indocyanine green given during a continuous infusion of BSP. Thirty-one min. before time zero 200 mg of BSP was administered intrav. as a priming dose. At the same time an infusion of 3.81 mg/min. was started and maintained throughout the study.

recovery of indocyanine green in the bile was 95.9% (Table I).

In dog 1, after a similar control period, a 45 mg dose of indocyanine green was followed by an infusion of 2.02 mg/min over the next 48 minutes (total quantity administered, 142 mg). Again the BSP plasma concentration rose sharply (from 2.9 to 4 mg %), then continued to rise very slowly throughout the indocyanine green infusion, after which it remained elevated and constant (Fig. 4). The

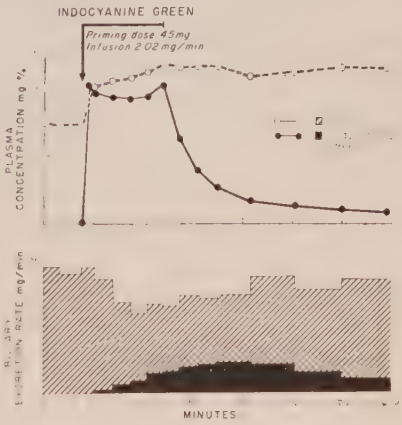

FIG. 4. Effect of combined infusions of indocyanine green and BSP. Forty-five min. before time zero 250 mg of BSP was administered intrav. as a priming dose. At the same time an infusion of 3.91 mg/min. was started and maintained throughout the study. Bile was collected for 3 hr beyond the time shown above in order to measure total recovery of indocyanine green.

biliary excretion rate of BSP appeared to be reduced during the period of maximum indocyanine green excretion. It rose thereafter, but since it never quite returned to the control level it is difficult to interpret this observation with certainty. Eventual recovery of indocyanine green from the bile in this study was 97.7% of total administered dose (Table I).

It is clear that indocyanine green interfered with hepatic uptake of BSP from the plasma in both dogs. However, this interference apparently was overcome by an elevation of the BSP concentration so that, in spite of the continued presence of indocyanine green, BSP removal rate from plasma again became practically equal to the infusion rate. It is understandable that this should occur since the removal of BSP from plasma does not appear to be a rate limited process (in contrast to biliary secretion), and rate of removal is normally accelerated whenever there is an increment in plasma concentration(4). Whether indocyanine green partially inhibits an hepatic BSP uptake mechanism, whether the 2 dyes compete for a common mechanism, or whether indocyanine green displaces BSP previously stored in the liver cannot be deduced from the present data. Although the last study (Fig. 4) suggests that indocyanine green may also interfere or compete with BSP in the biliary secretory process, further clarification of this and other possibilities must await the availability of much larger quantities of the new dye.

D. Intestinal absorption. Through the Thomas fistula 20 ml of aqueous solution containing 25 mg of indocyanine green was introduced into the duodenum of dog 2. Bile was collected over the next 4 hours. Low concentrations of indocyanine green were detectable in the bile after one hour, reaching a maximum during the third hour and diminishing thereafter. The total quantity recovered in 4 hours was 0.47 mg. or 1.9% of the administered dose.

To the same dog on another occasion 31 ml of bile (from the study on dog 1 described in the preceding section) containing 46 mg of indocyanine green, was administered into the duodenum. There was no detectable indocyanine green in the bile collected during the next 3 hours.

It is concluded that the intestinal absorption of indocyanine green is minimal and that reabsorption and recirculation of material excreted in the bile should introduce no significant error into studies undertaken without bile collection.

Summary. 1. Indocyanine green given intravenously to dogs is distributed in the plasma compartment and rapidly removed by the liver. 2. Biliary excretion of the dye is delayed, but an average of 97.3% of the administered dose is eventually recovered from the bile in apparently unaltered form. Indocyanine green does not appear in the urine. 3. Indocyanine green interferes with hepatic uptake of BSP from the plasma. 4. Absorption of indocyanine green from the bowel is minimal.

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Attempts to Produce Localized Shwartzman Reaction in Seven Species of Animals.* (24230)

Douglas E. Johnstone, Sol M. Michaelson, Lawrence Tuttle and Joe W. Howland

Department of Radiation Biology, University of Rochester School of Medicine and Dentistry, Rochester, N. Y.

When an intradermal injection of certain bacterial toxins is followed after 24 hours by intravenous injection of a similar toxin, a gross hemorrhage occurs at site of intradermal injection. Since the observation of this phenomenon by Shwartzman(1), the literature(2) on the subject infers that the reaction occurs in many species besides the rabbit. Increased interest in the Shwartzman phenomenon has come from a recognition that a parallel situation may exist in certain human disease states. Topley and Wilson(3) summed up this concept as follows: "The possibility that in natural infections there may be local alterations in the reactivity of tissues, particularly of vascular tissues, induced nonspecifically by substances of bacterial origin, cannot be overlooked in our attempts to analyze the phenomena of resistance to infection. Thus the hemorrhagic episodes in certain chronic infections of man may be related to the (Shwartzman) phenomenon. There is ample ground for speculation, but little direct proof."

The purpose of this study was to attempt to induce the localized Shwartzman reaction in several mammalian species.

Methods and materials. The following animals were used: 265 New Zealand albino male and female rabbits weighing 1.8-2.5 kg, 15 *Macacca mulatta* monkeys weighing 4-7 kg, 20 mongrel male and female dogs weighing 12-20 kg, 16 guinea pigs, 16 small swine (Hormel "minipigs") weighing 40-50 kg, 15 Wistar albino rats and 4 Toggenburg goats weighing approximately 30 kg. Three toxins were used to elicit the Shwartzman reaction: (1) meningococcal "agar washings toxin" filtered free of bacteria (supplied by Dr. Gregory Shwartzman), (2) partially purified "polysaccharide toxin" made from a pure culture of *Serratia marcescens*† and (3) a crude *Serratia marcescens* agar washing toxin, prepared by us from pure culture of this organism† by the same technic used in preparation of the meningococcal toxin(4). Each new preparation of toxin was assayed biologically to determine optimal dose and concentration necessary to give positive reactions in largest percentage of rabbits, while causing minimum lethality from the test itself. After this assay, the toxins were used in our tests. All 3 types of toxin were used in rabbits, monkeys and rats. Only the meningococcal toxin and our own *Serratia marcescens* toxin were used in dogs, minipigs, guinea pigs and goats.

The optimal dosage, previously worked out by biological assay in the rabbit, was given as a 0.50 ml intradermal dose, followed 24 hours later by intravenous dose of 1 ml of toxin. This dosage was used in the rabbits, rats, guinea pigs, goats, and monkeys. Ten dogs and 5 swine received 2.5 and 5.0 ml respectively for their intravenous dose. For further standardization of dosage in large animals, 10 additional dogs and 11 swine received intravenous doses comparable to that given rabbits (0.5 ml of a 1/40 dilution of toxin per kilo of body weight).

Results. Typical positive localized Shwartzman reactions occurred in 165 (62%) of 265 rabbits tested. No such reaction occurred in monkeys, dogs, rats, guinea pigs, goats, or minipigs tested (Table I). One of the goats, a post-partum lactating female, died several hours following intravenous injection of toxin. Although there was no local skin hemorrhage at site of intradermal injection, microscopic sections‡ of kidneys of this animal revealed a

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† Supplied by Dr. Murray Shear, Nat. Inst. Health.

‡ Dr. George Casarett reviewed the microscopic sections of goat kidney.

TABLE I. Incidence of Positive Localized Shwartzman Reactions in Various Species.

Species	No. of animals tested	Toxin used: *S. marcescens* filtrate	%	Meningococcal filtrate	%	*S. marcescens* polysaccharide	%
Rabbits	265	138/221	62	28/33	85	9/11	82
Monkeys	15	0/5	0	0/5	0	0/5	0
Dogs	20	0/15	0	0/5	0		
Goats	4	0/2	0	0/2	0		
Rats	15	0/5	0	0/5	0	0/5	0
Guinea pigs	16	0/10	0	0/6	0		
Minipigs	16	0/14	0	0/2	0		

"typical generalized Shwartzman reaction" (5), *i.e.* focal hemorrhagic cortical necrosis. Similar renal lesions were not found in animals of other species succumbing as a result of our test. No deaths or other untoward effects occurred in any of the groups of dogs, monkeys, minipigs, guinea pigs, or rats. Although the amount of toxin per kilo of body weight given the goat was markedly smaller than that given to rabbits, one goat which received 1 ml intravenously, developed a generalized Shwartzman reaction.

Discussion. Shwartzman(6) was able to induce the localized reaction in the golden Syrian hamster. He had previously reported (2) some success in inducing the phenomenon in guinea pigs after many unsuccessful attempts with the same species. He also mentions that Gratia and Linz(7) produced the reaction in guinea pigs, using 6-day-old *Vibrio cholerae* culture filtrate. Vassiliadis(8), using both cholera and El Tor vibrios, however, was unable to induce the Shwartzman reaction in guinea pigs. Gross(9) similarly failed, using *B. typhosa* filtrates. Freund(10) was also unable to produce the local hemorrhagic phenomenon in guinea pigs with *B. typhosa* preparations, but reported local skin hemorrhages in this species, if the animals received a skin-preparatory injection of diphtheria toxin, followed in 24 hours by intravenous injection of *B. typhosa* culture filtrate.

Generalized Shwartzman reactions (*i.e.* renal cortical hemorrhagic necrosis) were produced by Apitz(11) by means of a single intravenous injection of meningococcal toxin in pregnant rabbits. Thomas, *et al.*(5) confirmed this finding. Our experiments showed that a generalized Shwartzman reaction could be produced in a post-partum lactating goat.

Conclusions. 1. The localized Shwartzman reaction was produced in 62% of 265 New Zealand albino rabbits, using 3 different toxins (a meningococcal agar washings toxin, a partially purified "polysaccharide toxin" prepared from a culture of *Serratia marcescens*, and a crude *Serratia marcescens* agar washing toxin). Using these same toxins, localized Shwartzman reactions could not be induced in 15 *Macacca mulatta* monkeys, 20 mongrel dogs, 16 guinea pigs, 15 Wistar albino rats, 16 small swine (Hormel "Minipigs"), and 4 Toggenburg goats. 2. One post-partum lactating female goat developed a typical "generalized Shwartzman reaction" (*i.e.* renal cortical hemorrhagic necrosis).

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Prolongation and Potentiation of Oral Codeine Analgesia in the Rat.* (24231)

BERNARD A. BECKER AND EDWIN E. HAYS
Research Department, R. J. Strasenburgh Co., Rochester, N. Y.

Although codeine has only moderate oral analgesic properties, it has long been employed particularly in combination with aspirin, phenacetin, and caffeine compounds. Since even such mixtures must be given every few hours for prolonged pain relief, a preparation has been sought which would produce a longer acting and potentiated response. This study describes analgetic activity of codeine complexes and combinations having such properties. In view of findings that drug-resin products dissociate slowly in the G.I. tract causing delayed absorption and prolonged drug action(1,2), codeine has been reacted with synthetic ion exchange resins† to form a resinate complex which appears to have a prolonged duration of action. (Additionally, methylatropine and Tuazole (2-methyl-3-orthotolyl-4-quinazolone) have been found to potentiate the analgesic activity of codeine. Other quinazolones (including halogen derivatives) are under investigation. These drugs form reaction products with synthetic ion exchange resins which likewise show prolonged action *in vivo*.

Methods. The D'Amour and Smith(3) apparatus and technique for analgesiometric measurement in the rat was used with a 300 watt AC projection lamp at 95-100 volts as a heat source. Two groups of 10 animals each were placed in the dolorometer and their response time measured. The voltage was adjusted so that mean baseline response time was of the order of 2 sec. The voltage was then kept constant throughout the experimental period. The pre-treatment response time of each animal was measured 3 times. The average of second and third values was taken as the animal's baseline response time. Drugs were given by gavage as aqueous solutions or suspensions at a 5% dose volume. Response time was measured 30 min. after dosing and then at intervals usually 1 hour apart. Ten sec was the maximal stimulus time in any measurement. For each animal, baseline response time was subtracted from each post-dose response time (= Δ sec). This value less 3 Δ sec (the criterion of minimum analgesia) was multiplied by hours elapsed after administration of the dose (= Δ sec hr) and totaled (Σ Δ sec hr) for each animal. The mean and stand. dev. of Σ Δ sec hr of test and control groups were calculated. "Students t" test was used to demonstrate whether the difference of the means was statistically significant.

Results. Reproducibility of analgesic measurements. (1) Rat-to-rat variation is large as evidenced by large stand. dev. (2) day-to-day variation is greater than dose-to-dose variation (Table I). Accordingly, a reproducible log dose-effect curve could not be derived. Each test is a 1 x 1 direct comparison.

Codeine sulfate. 50 mg/kg of codeine showed effective analgetic response (greater than 3 Δ sec) at 30 min but not after 90 min. At 75 mg/kg potency was similar but effec-

TABLE I. Measurement of Analgesic Activity of Codeine Sulfate.

Dose, mg/kg (as codeine)	Date	$\bar{\Sigma}$ (Δ sec.–3) hr	No. of rats
50	7/15/57	7.64 ± 8.7	10
	9/ 3	1.79 ± 3.5	9
	6	5.53 ± 9.5	8
	13	1.78 ± 5.2	9
	10/ 3	2.15 ± 3.2	4
	11	17.73 ±20.0	8
	24	7.40 ± 7.7	3
	25	13.60 ±12.6	5
	12/ 4	2.82 ± 5.4	10
	3/ 3/58	17.21 ±13.1	10
	Mean	7.76	
75	7/ 2/57	17.88 ±20.3	5
	3	16.56 ±20.8	5
	Mean	17.17	

* A preliminary report of this work was given at the Fed. Am. Soc. Exp. Biol., April, 1958.

† The sulfonic acid cation exchangers Amberlite IR-120 and XE-69 were used.

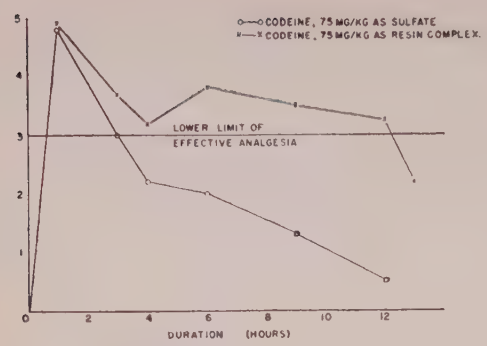

FIG. 1. CODEINE ANALGESIA IN THE RAT. PROLONGATION BY RESIN COMPLEX.

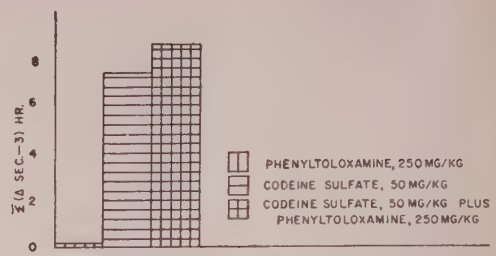

FIG. 2. EFFECT OF PHENYLTOLOXAMINE ON CODEINE ANALGESIA

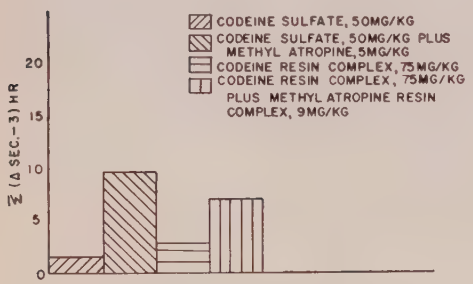

FIG. 3. POTENTIATION OF CODEINE ANALGESIA BY METHYL ATROPINE.

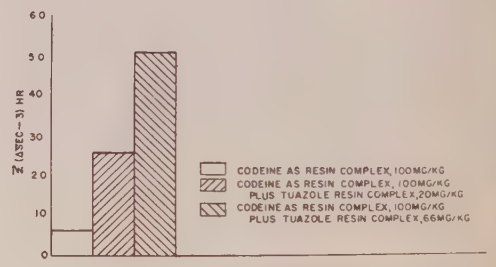

FIG. 4. POTENTIATION OF CODEINE ANALGESIA BY TUAZOLE.

FIG. 1-4.

tive analgesia lasted 90-180 min (Fig. 1).

Codeine resinate. 75 mg/kg produced effective analgesia equal in intensity to that of 50 mg/kg of codeine as sulfate, but of much longer duration (4-12 hr) than that of even 75 mg/kg of codeine as sulfate. Hence, prolongation of effect is attained.

Codeine potentiators. Phenyltoloxamine, coadministered in doses up to 250 mg/kg did not enhance the analgetic activity of 50 mg/kg of codeine as sulfate (Fig. 2). Methylatropine (Metropine‡) at 5 and 10 mg/kg as the nitrate enhanced the analgetic action of 50 mg/kg of codeine as sulfate. 9 mg/kg of methylatropine as resinate potentiated the analgetic effect of 75 mg/kg of codeine as resinate (Fig. 3). Tuazole, which has no analgetic properties, in a dose of 20 mg/kg increased the analgetic activity of 50 and 100 mg/kg of codeine as sulfate. When both codeine (100 mg/kg) and Tuazole (20 mg/kg) were given as resinates, the analgesic activity was increased by an average factor of 4.3. Increase of the Tuazole dose to 66 mg/kg increased the factor to 6.4 (Fig. 4).

‡ R. J. Strasenburgh Co. registered trademark for methylatropine nitrate.

Discussion. Prolongation of therapeutic activity of a number of drugs has now been achieved by reacting them with appropriate synthetic ion exchangers(1,2). Where the drug resinate exhibits prolongation of action, it is less toxic than the soluble salt form when compared on a "free" drug basis(4).

The D'Amour-Smith rat analgesiometric technic as a means of predicting analgesic drugs has been criticized because of the difference in metabolism of codeine in rat(5) and man, and the persistence of the tail flick reflex after spinal section. Nevertheless, Beecher concluded that the D'Amour-Smith technic was useful in evaluation of potential analgesics(6).

There is no agreement as to treatment of data obtained by the D'Amour-Smith technic. Some workers employ an all-or-none basis for

evaluation while others treat data as graded responses(7). We have employed a combination of quantal and graded response criteria. Three Δ sec change in response time is used as an all-or-none criterion for establishment of adequate analgesia. Pain in man is a graded sensation. Therefore, it would appear reasonable to treat responses greater than 3 Δ sec in the rat as graded responses. The use of a 10 sec cut-off time introduces bias into the treatment of graded responses(8). However, values above 10 sec would be interpreted as having greater analgetic potency and thus in this case the bias increases conservatism in predicting the efficacy of new analgetics.

Duration, as well as intensity of effect, becomes a problem in evaluation of long-acting drugs. Winter(9) has suggested a simple means. However, the calculation employed above is considered an improvement in that it gives greater weight to duration than to degree of analgetic.

Successful potentiation of oral codeine analgesia by non-analgetic drugs has not, to authors' knowledge, previously been accomplished. Prostigmine was claimed to potentiate codeine but this finding was not confirmed. Solanaceous alkaloids have been claimed to depress intensity and duration of morphine effects(6). The evidence presented here shows that methylatropine potentiates codeine analgesic. The combinations of codeine with methylatropine and with 2-methyl-3-orthotolyl-4-quinazolone, all as ion exchange resin complexes are now undergoing clinical evaluation. Preliminary reports indicate that each of the combinations possesses enhanced analgetic potency and duration of effect(10).

Summary. 1. The reaction product of codeine and appropriate synthetic ion exchange resins has been demonstrated to possess analgetic properties of codeine. The resin-complex has a longer duration of action than does soluble forms of the drug. 2. Codeine analgesia has been potentiated by methylatropine and 2-methyl-3-orthotolyl-4-quinazolone. Both the resin complex and soluble salt forms of codeine are potentiated by these agents. 3. Resin reaction products of methylatropine and 2-methyl-3-orthotolyl-4-quinazolone have been prepared. Both of the drug resin combinations exhibit prolongation of action, including the property of codeine potentiation, the latter drug beng more potent in this respect. 4. Combinations of codeine-resin complex and of methylatropine or 2-methyl-3-orthotolyl-4-quinazolone in resin complex form are potent, long acting analgesics in the rat. 5. Analgesiometric data obtained in the rat by the D'Amour-Smith method have been treated so as to express both duration and intensity of action. More weight is given to duration of adequate analgesia than to intensity of analgesia.

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Effect of Antioxidants on Dietary Necrotic Liver Degeneration.[1] (24232)

KLAUS SCHWARZ
National Institute of Arthritis and Metabolic Diseases, N.I.H., Bethesda, Md.

Necrotic liver degeneration is readily produced in rats by *Torula* yeast diets which are simultaneously deficient in cystine*, Vit. E, and Factor 3(1,2). Since the disease is prevented by physiological amounts of tocopherol (3,4), it belongs to those Vit. E deficiency syndromes which show extensive anatomical damage and which are fatal. Other such diseases are encephalomalacia, exudative diathesis, muscular dystrophy, and acute heart degeneration(5).† It is not fully known what causes Vit. E deficiency to document itself in such a wide variety of pathological lesions. However, it is certain that the supply of Factor 3-selenium‡ has a decisive effect on the pathology produced by Vit. E-deficient diets.

In vitro, various forms of Vit. E are effective as antioxidants, although greatly divergent in biological potency(6,7). This has been the basis for much conjecture as to their mode of action *in vivo,* without leading to an accepted, clearly understood concept for the biological mechanism of action of Vit. E(8). The present paper deals with effects of various groups of antioxidants on dietary necrotic liver degeneration. A total of 13 such compounds was tested. Liver necrosis was found very effectively prevented by a few of these substances, but not by others.

Methods. Assays reported here were carried out on rats of the Fischer 344 strain(9).§ This albino strain has been brother-sister mated through more than 60 generations and is very uniform in responding to the basal, liver necrosis-producing diet. Under our standard conditions, Fischer animals require an average of 21 days for development of liver necrosis, as compared to 45 days for the Sprague-Dawley strain referred to previously. For protection against liver necrosis, Fischer rats need only approximately half as much dietary Vit. E as Sprague-Dawley rats. Necrotic liver degeneration was produced by a Vit. E-free, 30% *Torula* yeast diet, as described previously(1). The predepletion method was used(2). Supplementation of the diet with the various antioxidants started after the animals had been kept for 12 days on the basal ration. In all experiments, unsupplemented groups of rats were maintained as controls. Food and tap water were given *ad libitum*. Supplements were either added directly to the whole diet (D), or suspended in Vit. E-free lard before incorporation of the latter in the ration (L). Diets were made up in 1 or 2 kg lots and fed in porcelain cups holding approximately 80 g; these were checked daily. Unused diets were stored at +4°C. All animals were autopsied, and a few animals which died from causes other than liver necrosis were omitted. The results were evaluated by comparison of numbers of survivors, and also of survival times. The latter were used for calculation of average velocity (V_{st} = 100/survival time) of death from liver necrosis, as described previously (3,10). For estimation of the protection caused by a supplement, the V_{st} of the supplemented group was compared to that of the controls. "Per cent protection" was calculated by expressing the reduction of V_{st}, resulting from the supplement, in per cent of

[1] This manuscript was originally submitted on March 16, 1956.

* Since submission of this paper it has been shown that Factor 3 is an organic selenium compound (Schwarz, K., Foltz, C. M., *J. Am. Chem. Soc.,* 1957, v79, 3292). The protective effect of L-cystine is caused by a trace contamination with Factor 3-active selenium. To be published.

† Multiple necrotic degeneration (heart, liver, kidney and muscle necrosis) in the mouse also belongs into this category. (DeWitt, W. B., Schwarz, K., *Experientia,* 1958, v14, 28).

‡ Various selenium compounds show greatly different biopotencies (Schwarz, K., Foltz, C. M., *J. Biol. Chem.,* 1958, v233, 14). The term "Factor 3" is applied to designate the biologically active, selenium containing substance, or substances, in material of natural origin.

§Cooperation of the Animal Production Section, NIH, is gratefully acknowledged.

the V_{st} of the unsupplemented controls (% protection = 100 − (100 X V_{st} exp.) V_{st} cont.). This method permits one to express, in mathematically correct terms, protective effects obtained with groups of animals. It is more accurate than the simple comparison of numbers of survivors, since it gives consideration to length of survival of individual

TABLE I. Effect of Antioxidants on Dietary Necrotic Liver Degeneration.

Supplement: Dose (g/100 g of diet)	Supplement: Mode of suppl.*	No. of exp.	No. of animals	No. of survivors†	Duration (days)	V_{st}, avg velocity‡	% protection§
			Ascorbic acid**				
.0	C	2	20	1		5.2 ± .21–.6	
.5	D	1	10	2	30	3.4 ± .5	33
2.0	D	1	10	1	30	3.7 ± .5	28
			Methylene blue chloride††				
.0	C	1	10	1		4.3 ± .6	
.1	L	1	10	0		4.9 ± .1[a]	
.2	L	1	9	3		2.8 ± .5	35
.5	L	1	10	2	35	2.7 ± .4	36
1.0‖	L	1	—	—	35		
			Di-tert-amylhydroquinone‡‡				
.0	C	1	8	0		5.2 ± .3	
.25	D	1	10	9	30	.3 ± .3	93
			Santoquin§§				
.0	C	3	29	2		4.5 ± .4–.6	
.10¶	L	2	20	0		3.8 ± .1	(16)
.25	L	2	19	12	35	1.2 ± .3–.4	73
			Santoflex B‖‖				
.0	C	3	25	2		4.5 ± .5–.6	
.005	L	1	10	0		4.3 ± .3	
.05	L	1	10	1		3.2 ± .4	28
.10	L	2	20	1		3.6 ± .1–.5	19
.25	L	2	20	2	40	3.5 ± .3–.6	22
			DPPD (N,N′-diphenyl-p-phenylenediamine)¶¶				
.0	C	4	35	0		5.2 ± .2–.7	
.00062¶	L	1	10	0		4.8 ± .2	
.00125	L	2	20	0		3.4 ± .1–.2	34
.0025	L	1	9	0	42	2.8 ± .1	47
.0050	L	2	20	13	42	.6 ± 0–.2	89
.0200	L	1	10	10	60	0	100
.0	C	3	25	2		5.0 ± .5–.7	
.00125	D	1	8	1		3.4 ± .1	32
.0025	D	2	19	1	42	2.5 ± .3	50
.0050	D	1	10	10	42	0	100
			D,L-α-tocopheryl acetate				
.0	C	2	16	1		5.1 ± .5–.6	
.00125	L	1	9	8	42	.6 ± .6	88
.0025	L	1	9	9	42	0	100

* C = control, D = added directly to diet, L = premixed in lard before addition to diet.

† If not specially indicated, experiments were terminated between 25th and 35th day.

‡ Mean ± stand. error. Where several groups are combined, lowest and highest stand. error are listed.

§ $100 - \frac{V_{st}\ \text{exp.} \times 100}{V_{st}\ \text{control}}$.

‖ Toxic.

¶ Lower levels tested and found inactive.

** USP, Merck and Co.

†† National Aniline Division, Allied Chemical and Dye Corp.

‡‡ 2,5-di-tert-amylhydroquinone, Eastman Kodak Co.

§§ 6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline, Monsanto Chemical Co.

‖‖ 6-Phenyl-1,2-dihydro-2,2,4-trimethylquinoline, Monsanto Chemical Co.

¶¶ Food grade, B. F. Goodrich Chemical Co.

animal, and permits calculation of averages and statistical treatment of the data where survival times themselves cannot be averaged, due to animals which are maintained alive. With increasing duration of survival, velocity of death approaches zero (V_{st} of survivors $\longrightarrow$ 0).

Results. Results are presented in Table I. Data obtained in different experiments were pooled for each antioxidant, and control groups pertaining to tests of each substance were also combined. Growth was not significantly affected. Ascorbic acid and methylene blue afforded a small measure of protection in that they somewhat delayed development of the disease. However, rather high levels of the supplement were required; the effects were relatively small and seemed to reach a ceiling, so that with elevated doses no further increases in protection were obtained. The effect of 2% ascorbic acid in the diet was practically identical to that of .5%. Methylene blue protected 3 out of 9 animals when supplied at a .2% level. With .5% of the dye, 2 out of 10 animals survived; higher levels were clearly toxic.

The following substances were without significant influence. All were tested at dose levels up to .5% in the diet:

Antabuse (tetraethylthiuram disulfide)‖
Hydroquinone¶
NDGA (nordihydroguaiaretic acid)**
n-Propylgallate (propylester of gallic acid)††
DBPC (2,6-di-tert-butyl-4-methyl-phenol)‡‡
Butylated hydroxyanisole§§
Propylparasept (propylester of p-hydroxy-benzoic acid)‖‖

The last 5 of these compounds are commercial, widely used, multiple-purpose antioxidants of phenolic character.

‖ Purified, Ayerst Laboratories, Inc.
¶ Mallinckrodt Chemical Works.
** Nordigard Corp.
†† Purified, Heyden Chemical Corp.
‡‡ Food grade, Koppers Co., Inc.
§§ Mixed 2- and 3-tert-butyl-4-methoxyphenol, Tenox BHA, Eastman Kodak Co.
‖‖ Purified, Heyden Chemical Corp.

Hydroquinone (.5%) was without effect but di-tert-amylhydroquinone prevented liver necrosis almost completely at a level of .25%. Effects were also seen from 2 dihydro-quinoline derivatives: Santoquin at a .1% level delayed onset of the disease, and .25% protected 12 out of 19 animals for more than 35 days. Santoflex B, only slightly different from Santoquin in its constitution, was much less active and showed a ceiling in its effectiveness.

Most conspicuous results were obtained with N,N′-diphenyl - p - phenylenediamine (DPPD). Activity of this antioxidant was of the same order of magnitude as that of D,L-α-tocopheryl acetate. Doses of 1.25 and 2.5 mg% had a pronounced delaying action on development of the disease. Out of 20 animals receiving 5 mg %, 13 survived for an experimental period of 42 days. When .02% DPPD was furnished in the diet all 10 animals survived; they were killed after 60 days. There was essentially no difference between diets prepared by mixing of the DPPD into lard before addition to the diet, and 2 groups which received 2.5 and 5 mg % of DPPD supplemented by addition directly to the complete basal ration. Control experiments with D,L-α-tocopheryl acetate listed in Table I were run simultaneously with some of the DPPD experiments.

Discussion. The present studies evidently deal with those aspects of necrotic liver degeneration which are related to Vit. E. This is also apparent from a subsequent paper, describing the effects of intraportal injection of antioxidants on respiratory decline(11). The effects of antioxidants such as methylene blue, Antabuse, ascorbic acid, and nordihydroguaiaretic acid (NDGA) on various experimental Vit. E deficiency diseases in chickens and rats were initially investigated by Dam and collaborators, as reviewed elsewhere(12,13). Liver necrosis was reported to be delayed by several of these substances. Moore, *et al.*, extended Dam's studies by testing a series of known oxidation-reduction indicators, such as dyes related to methylene blue, in Vit. E-deficient rats(14,15). Singsen, Jungherr, and collaborators subsequently detected that some commercial antioxidants prevented encephalo-

malacia in chickens. DPPD was practically as active as α-tocopherol(16). Contrasted with these findings is the observation that exudative diathesis, another form of Vit. E deficiency in the chicken, is much less responsive to DPPD supplements(17).

From the results presented here it follows that dietary necrotic liver degeneration belongs to those Vit. E deficiency diseases which are preventable by certain antioxidants, but various antioxidants are widely different in their effects. There is no apparent correlation between antioxidant activity and potency seen against liver necrosis. Roughly, the tested substances can be divided into 3 different groups: (I) Ascorbic acid, methylene blue, and Antabuse. These may act as antioxidants, but are better known for other properties. Antabuse is inactive at .2%, while the other 2 are required in rather large doses and afford only a limited, but measurable amount of protection. The results with methylene blue confirm those of Dam and Granados(18). In our experiments the effect does not surpass the 30 to 40% level of protection, even when increased levels of these agents are supplied. (II) A group of 5 antioxidants which are most widely and effectively used for stabilization of dietary fats and other materials, and which are without influence on necrotic liver degeneration at the tested levels. These antioxidants all have phenolic properties. (III) Antioxidants which are active, namely, di-tert-amylhydroquinone, Santoquin, and especially DPPD. These are chemically not related to each other. It is at present not possible to define the specific properties which enable them to protect, like Vit. E, against dietary necrotic liver degeneration. Singsen, Jungherr, and collaborators have tested a variety of antioxidants against exudative diathesis in chickens(16), and have reported a scale of relative activities similar to that described here. The only substance obviously different is DBPC, which is active in the chicken, but inactive in the rat.

It is inferred that the protective compounds function within the tissues and that the results are not simply due to stabilization of the diet against rancidity. This conclusion is warranted in spite of the fact that *Torula* yeast has been reported to contain several per cent of unsaturated fatty acids(19), for the following reasons: (1) Autoxidation of the diet is not involved in production of necrotic liver degeneration. The basal diet is stable against rancidity; this is due to the presence of antioxidants in the yeast(20); (2) The deficiency can be produced readily by fat-free rations(21)¶¶; (3) If a general antioxidant action against rancidity of the diet were involved, the 5 inactive antioxidants listed in group II should be effective. Evidence will be presented that the active antioxidants, when injected intraportally, are like Vit. E, effective *within* the liver tissue in reverting respiratory decline in the Warburg apparatus (11).

Summary. Thirteen antioxidants were tested at varying dose levels against dietary necrotic liver degeneration produced in rats by a Vit. E-deficient, 30% *Torula* yeast ration. The substances can roughly be grouped in 3 different classes: (I) Ascorbic acid (.5%) and methylene blue (.5%) showed activity, but did not afford more than 30 to 40% protection, even at higher dose levels. Antabuse (.2%) was ineffective. (II) Inactive antioxidants: NDGA (nordihydroguaiaretic acid), n-propylgallate, DBPC (2,6-di-tert-butyl-4-methylphenol), BHA (mixed 2- and 3-tert-butyl-4-methoxyphenol), hydroquinone, and others were without effect at dose levels up to .5% in the diet. (III) Antioxidants protecting against liver necrosis: Di-tert-amylhydroquinone and Santoquin (6-ethoxy-1,2-dihydro - 2,2,4-trimethylquinoline) inhibited the deficiency strongly at .25% levels in the diet, while Santoflex B (6-phenyl-1,2-dihydro-2,2,4-trimethylquinoline, .05 to .25%) was slightly active. DPPD (N,N′-diphenyl-p-phenylenediamine, food grade) came close to Vit. E in activity. 5 mg % DPPD were approximately as effective as 1.25 mg % D,L-α-tocopheryl acetate.

Acknowledgement is made to Esther M. Hurley and Roswell A. Taylor, Jr., for efficient technical

¶¶ Incidence and velocity of development of liver necrosis are unchanged when fat is eliminated from our basal *Torula* yeast diet.

assistance. For samples of antioxidants we are indebted to Ayerst Laboratories, N. Y., Nordigard Corp., Chicago, Heyden Chemical Corp., N. Y., Koppers Co., Pittsburgh, Eastman Chemical Products, Kingsport, Tenn., Shell Chemical Corp., N. Y. Monsanto Chemical Co., St. Louis, and the B. F. Goodrich Chemical Co., Cleveland, O.

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Effect of Cortisone and Growth Hormone upon Ductular Cell Proliferation in Liver Ethionine Intoxication.* (24233)

ROBERT J. STEIN, GEOFFREY KENT† AND HANS POPPER
Abbott Laboratories, Research Division, N. Chicago, Hektoen Inst. for Medical Research of Cook County Hospital, Chicago, Ill., and Dept. of Pathology, Mount Sinai Hospital, N. Y. City

Recently interest was focused upon accumulation of interstitial cells between liver cell plates, as apparent in biliary obstruction and dietary disorders(1), and in intoxication with carcinogenic drugs(2). These interstitial cells have been considered to be epithelium of bile ductules(2). Injection of bile ducts demonstrated that interstitial cells are to a large extent bile ductular cells even though morphologically they resemble mesenchymal elements (3). The interstitial cell reaction has been considered a response of the liver to injury, and this raised the question whether the reaction can be suppressed by anti-inflammatory agents, such as cortisone. Since this reaction can be elicited with reproduceable regularity in ethionine intoxicated rats(4), the influence of simultaneous cortisone administration was studied in view of known effect of cortisone upon inflammatory reactions in the liver(5,6). For comparison the effects of administration of growth hormone upon ethionine intoxication was also studied because of its influence on liver cells(7) and its antiphlogistic effect possibly antagonistic to cortisone(8).

Methods. Female Sprague-Dawley rats weighing approximately 175 g were placed in individual cages for 25 to 35 days and kept on synthetic diet containing vitamin free case-

* Supported, in part, by Grant No. C-2030, United States Public Health Service, National Institute of Health.

† Present address: West Suburban Hospital, Oak Park, Ill.

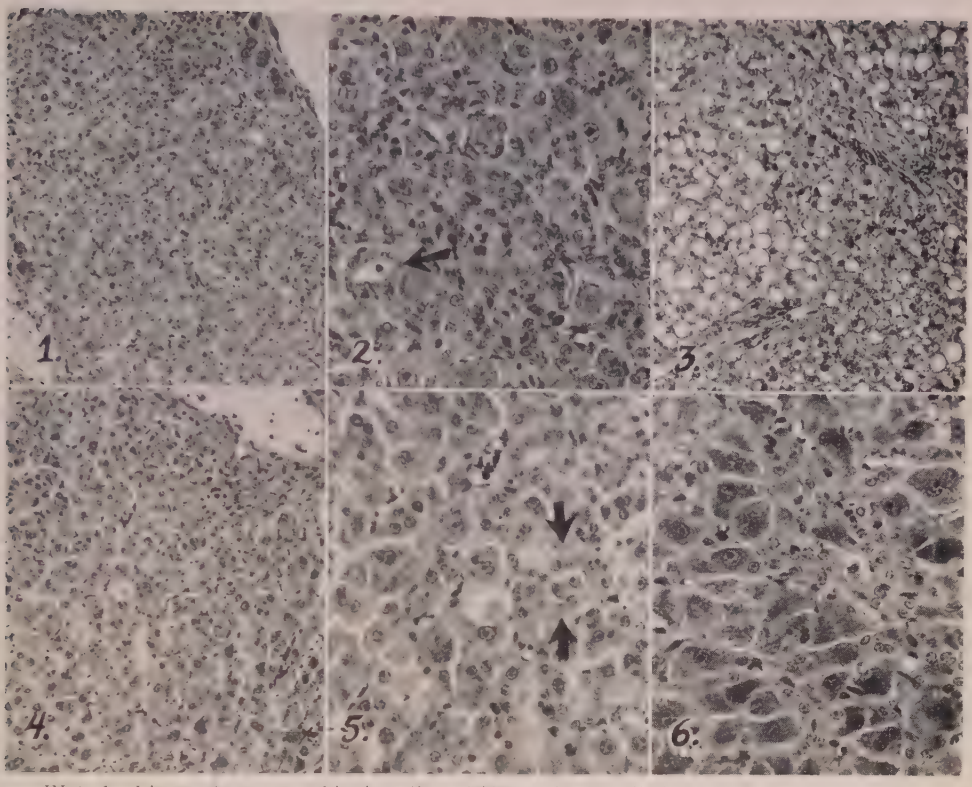

FIG. 1. Liver of rat on ethionine diet. Diffuse single cell degeneration and regeneration and interstitial cell reaction. H & E. 80×.

FIG. 2. Liver of rat on ethionine diet. Interstitial cells consist of ductular cells occasionally arranged around lumina (arrow) intermixed with inflammatory cells including segmented leucocytes. H & E. 160×.

FIG. 3. Liver of rat which had received cortisone. Severe diffuse fatty metamorphosis. H & E. 80×.

FIG. 4. Liver of rat on ethionine diet which had received cortisone. Liver damage is the same as in rats not receiving cortisone. However, the interstitial cell reaction is almost entirely absent. H & E. 80×.

FIG. 5. Liver of rat on ethionine diet which had received cortisone. In places (arrow) transitional stages between liver cells and ductular cells are noted. H & E. 160×.

FIG. 6. Liver of rat on ethionine diet which had received growth hormone. The interstitial cell reaction is almost as severe as in rats not receiving growth hormone and the liver cells show damage but no fatty metamorphosis which is seen in rats on growth hormone in the absence of ethionine administration. H & E. 160×.

in, 160 g; corn oil, 50 g; sucrose, 750 g; salt mixture U.S.P., for depletion diet, 40 g/kilo; and adequate vitamin supplements, except for riboflavin, 4 mg; and choline hydrochloride, 30 mg. One group of 10 rats, serving as control, were on the basal diet. A second group of 20 rats had a dietary supplement of 0.5% ethionine. A third group of 10 rats on basal diet received 5 mg of cortisone intramuscularly 3 times a week. A fourth group of 25 rats on ethionine supplemented diet had the same amount of cortisone as Group 3. A fifth group of 10 rats on basal diet received 0.5 mg of growth hormone‡ (Armour and Company's "SOMAR," lot no M208) intramuscularly 6 days/week. A sixth group of 20 animals were on ethionine diet while receiving same amount of growth hormone. At completion of the experiment, the animals were

‡ The authors are indebted to Dr. Irby Bunding of the Armour Laboratories, Chicago, for supplying the growth hormone. It was crystallized in a lyophilized form. Prior to use it was reconstituted with triple distilled water. Portions of rehydrated hormone not used immediately were frozen at -20°C, then thawed at room temperature prior to experimental use.

TABLE I. Effect of Cortisone and Growth Hormone on the Rat Liver in Ethionine Intoxication.

Procedure	No. of rats	Duration (days)	Parenchymal degeneration	Cell regeneration	Fatty metamorphosis	Interstitial cells			
						Ductular	Inflamm.	Collagen	Reticulum
Basal diet, 7 d/wk	10	30	0	0	2.0	0	0	0	0
.5% ethionine in basal diet, 7 d/wk	20	30	2.0	4.0	0	4.0	1.0	0	4.0
Cortisone, 5 mg IM 3× /wk + basal diet, 7 d/wk	10	30	0	0	1.0	0	0	0	
Cortisone, 5 mg IM 3× /wk + .5% ethionine in basal diet, 7 d/wk	25	30	1.0	2.0	.25	.25	Nil	0	1.0
Growth hormone, .5 mg IM 6 d/wk + basal diet, 7 d/wk	10	30	0	0	4.0	0	0	0	0
Growth hormone, .5 mg IM 6 d/wk + .5% ethionine in basal diet, 7 d/wk	20	30	1.0	2.0	.25	2.0	1.0	0	2.0

sacrificed and their livers were subjected to routine histologic analysis. The histologic study was done without knowledge of experimental history and individual morphologic findings were graded from 0 to 4+ to be subsequently averaged for the entire group.

Results. Rats on basal diet exhibited a varying but slight degree of fatty metamorphosis and showed occasionally focal necrosis associated with accumulation of mononuclear cells. Animals on ethionine in the diet showed severe single cell degeneration and regeneration characterized by focal ballooning and coagulation of cytoplasm on the one hand and presence of very large vesicular nuclei with prominent nucleoli on the other (Fig. 1 and 2). The liver cell plates were widely separated, throughout the lobule, by accumulation of cells with vesicular, round or oval nuclei and relatively little cytoplasm. In previous studies these cells were shown to be ductular cells(3) since occasionally they surrounded thin lumina and connected with bile canaliculi or portal bile ducts. The ductular cells were intermixed with unquestionable inflammatory cells including segmented leucocytes. The sinusoidal lumen appeared obstructed. Argentaffine fibers were conspicuously increased but collagenous fibers were virtually unaltered (Table I).

Rats receiving cortisone with the basal diet exhibited moderate fatty metamorphosis (Fig. 3) far in excess of that seen on the basal diet alone. In rats on ethionine and cortisone, fatty changes were minimal but liver cell alterations were of almost equal degree as in ethionine fed rats. The ductular cell reaction in contrast was almost entirely absent (Fig. 4) and the enlarged and damaged liver cells were closer to each other. The capillaries were clearly recognizable and the Kupffer cells were enlarged. The ductular cell reaction was noted only in portal spaces and about foci of eosinophilic necrosis. The cytoplasm of numerous cells in these areas appeared slightly basophilic and more abundant than in ductular cells, creating a resemblance to liver cells and suggesting a transition between ductular cells and hepatic epithelial cells. Mononuclear inflammatory cells and segmented leukocytes were entirely absent (Fig. 5). Silver impregnations exhibited considerably fewer argentaffine fibers than were seen in non-cortisone treated rats on ethionine diets.

Rats on basal diet receiving growth hormone exhibited a severe fatty metamorphosis particularly on the lobular periphery. In contrast, in rats receiving ethionine and growth hormone, fatty metamorphosis was not conspicuous and the picture was similar to that of rats receiving ethionine alone (Fig. 6) except that epithelial cell damage and ductular cell reaction were less intense. Distribution of the latter was less diffuse and the cells revealed a

tendency to arrange themselves around lumina. In principle, the alterations in this group resembled far more those produced by ethionine alone than by ethionine with cortisone.

Discussion. The repeatedly described alterations of liver produced by ethionine(4,2) appeared in this series particularly early and regularly, probably because use of single cages equalized food intake. The alterations are characterized by hepatocellular and interstitial cell changes. Cortisone distinctly suppresses both components of the interstitial cell reaction by practically eliminating the inflammatory as well as bile ductular cells. The anti-inflammatory effect of cortisone is well established(9). Suppression of the diffuse ductular cell reaction in the liver deserves emphasis and has to be compared with suppression of other epithelial cell reactions(10-13). In circumscribed areas such as portal tracts or foci of necrosis. cells are seen which suggest a transition betwen liver cells and ductular cells. In contrast to previously held belief, it appears now established(14) that in the embryo liver cells develop into ductular cell reaction(2). However, under cortisone the direction of the transition under cortisone also goes from liver cells into ductular cells. One could speculate that under the influence of cortisone such a transition is more conspicuous because of retardation of the process, and may represent a response to injury.

The previously demonstrated(3) relation between ductular cells and reticulum fiber proliferation is confirmed by the smaller number of reticulum fibers in cortisone-treated ethionine rats. The liver cell damage has been considered an effect of interstitial cell reaction(2). However, under cortisone treatment they are clearly dissociated. The simultaneous disappearance of ductular and inflammatory cell reaction suggests that they are produced by the same stimulus or that the ductular reaction may induce an inflammatory cell response.

The fatty metamorphosis present to a moderate degree in rats on basal diet or on cortisone, and to a very conspicuous degree in rats on basal diet supplemented by growth hormone, is absent in ethionine intoxicated rats even with the supplements above. This lipotropic effect may be the result of marked metabolic disturbance created by ethionine comparable to the lipotropic effect of reduced metabolic needs in starvation(15). The effect of growth hormone itself is not conspicuous. It does not exaggerate the interstitial cell reaction but, if anything, mitigates it to produce a low-grade cortisone effect, perhaps explained by its corticotropic effect or cortisone-growth hormone antagonism(16).

Summary. Administration of cortisone inhibits accumulation of interstitial cells between liver cell plates, otherwise produced by oral administration of ethionine to rats. The inflammatory component of interstitial cells consisting of mononuclear cells and segmented leucocytes is entirely abolished whereas the otherwise prevalent ductular cell reaction, also considered a response of liver to injury, is almost completely prevented. Where ductular cells form, transitions between these and liver cells are observed. Liver cell degeneration characteristic of ethionine intoxication is not influenced by cortisone. Administration of growth hormone has a greatly reduced cortisone effect. Ethionine intoxication prevents the fatty metamorphosis produced by administration of cortisone and growth hormone.

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Fractionation of Cell Types in Ehrlich Ascites Fluid by Sucrose Gradient Centrifugation.* (24234)

A. O. HAWTREY (Introduced by A. Polson)
Liesbeek Cancer Research Clinic, Liesbeek Road, Rosebank, Cape Town, S. Africa

For studies in our laboratories concerning the influence of X-irradiation on cell metabolism, it was necessary to obtain a supply of Ehrlich Mouse Ascites Tumor Cells free from erythrocytes, leucocytes and other cell types usually present in ascites fluid.

Erythrocytes may be removed by lysis with hypotonic saline which appears to leave ascites cells intact(1,2). Centrifugation of the mixed cells in Krebs-Ringer bicarbonate or isotonic saline affords a rough separation between erythrocytes and tumor cells whereby either layer may be scooped out with a glass spatula (3). The counter-streaming centrifugal procedure of Lindahl and Klein(4) yields considerably purified tumor cell preparations, but none in which contaminants are completely absent.

We have shown that centrifugation of the mixed cells through a sucrose gradient affords a separation into 4 distinct bands of different cell types. One of these appears to consist of pure ascites tumor cells which are viable.

Methods. Fractionation of cell types. A suitable sucrose gradient was prepared in a centrifuge tube (17 x 3 cm diameter). After first filling with 0.07 M phosphate buffer of pH 7.4, a layer of 50% w/v sucrose in phosphate buffer was introduced into the bottom 2 cm of the tube using a long Pasteur pipette with a small upturned tip. Successive 1 cm layers of sucrose solutions in phosphate buffer, each solution 5% w/v less in concentration than the previous one, were then introduced by upward displacement through the Pasteur pipette until a concentration gradient of 10-50% w/v of sucrose was obtained. After standing 15-30 minutes a glass rod was moved gently up and down through the solution to ensure an even gradient. Mouse ascites fluid (10 ml) was mixed with sodium citrate anticoagulant solution on withdrawal, then centrifuged. The cell mass (2-3 g wet wt) was twice washed in isotonic saline at 5°C and finally resuspended in 10 ml saline. A portion of the cell suspension (1.5-2.0 ml) was carefully layered on top of the sucrose gradient, and the tube centrifuged at 250-300 rpm for 30 minutes at 10-15°C.

Viability tests. Separate experiments were carried out to test the effect of strongly hypertonic (1.17 M) 40% sucrose solution on the viability of ascites tumor cells.

1. *Oxygen uptake of ascites cells treated with 40% sucrose solution in phosphate buffer.* A suspension of saline-washed mixed ascites cells was prepared in phosphate buffer containing 40% w/v sucrose. The suspension was divided into 2 portions, one of which was allowed to stand 40 minutes at 23°C,

* The work was conducted at the instigation of Professor J. van Rooijen. I am indebted to Dr. A. Polson for suggesting the use of a sucrose gradient, to Dr. G. de Kock for microscopical identification of the cells, to Dr. M. H. Silk and Mr. I. J. C. Macintosh for manometric measurements, and to Mr. H. F. Montauban van Swijndregt for valuable technical assistance. The Ehrlich ascites tumor was originally supplied to us by favour of Dr. Kanematsu Sugiura of the Sloan-Kettering Institute.

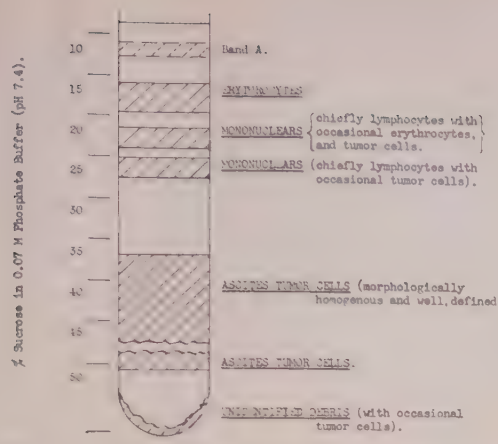

FIG. 1. Fractionation of cell types present in Ehrlich mouse ascites fluid by means of centrifugation in a sucrose gradient.

and the other for the same time at 0°C. Thereafter both portions were dialysed for 1½ hours at 4°C against 0.15 M sucrose (5% w/v) in phosphate buffer. The cells were removed from residual sucrose solution by centrifugation, after which they were resuspended in a medium consisting of mouse ascites serum previously neutralized with hydrochloric acid, buffered with 0.03 M phosphate to pH 7.4 and fortified with 0.25% w/v d-glucose. The final suspension contained 40 mg of sucrose-treated cells/1 ml of fluid and was used for measurement of oxygen uptake in Warburg manometers at 37°C.

2. In vivo test of viability of ascites cells treated with 40% sucrose in phosphate buffer. In addition to measurements of respiratory function, the viability of 40% sucrose treated cells was tested *in vivo*. Portions (0.1 ml) of the final suspension of treated cells in phosphate buffered and glucose fortified ascites serum were inoculated into mice under conditions used for routine transplantation of Ehrlich Ascites tumor.

Results. After centrifugation the sucrose gradient was examined against the light. Four distinct bands of cells were revealed, together with some coarser material deposited at bottom of the tube. Microscopic examination of the bands was carried out, following their careful removal with a sampling pipette. The composition of the various layers is illustrated in Fig. 1.

In some runs a thin white band (Band A—Fig. 1) of unidentified material was seen above the erythrocyte layer. The main bulk of ascites cells was found to sediment at the 40% sucrose level. In certain cases continued centrifugation (15-30 minutes) resulted in separation of a further narrow band of ascites cells from below the main bulk, indicated by a broken line at the 45% sucrose level in Fig. 1.

Ascites cells removed from the sucrose gradient appeared to be intact. The cell membrane was somewhat crinated and ratio of cytoplasmic to nuclear volume reduced owing to a certain amount of dehydration. The cells appeared to be more intensely stained by haematoxylin-eosin than fresh untreated ascites cells.

Although large mononuclears were probably present in the main band of ascites cells, these were not directly sought by means of the special technics required to distinguish them from ascites cells.

The respiratory function of ascites cells was apparently not impaired after contact with strongly hypertonic 40% w/v sucrose. The Qo_2† of the cells maintained for 40 minutes in 40% w/v sucrose at 23°C was −5.1 while that of cells maintained for the same time at 0°C was −5.4. Both values are within the limits of −5.0 to −10.0 quoted by Warburg(5) for ascites cells metabolizing in ascites serum, and would indicate that viability of the cells is not affected by contact with 40% w/v sucrose for the time required during centrifugation.

Tumors were produced in 100% of mice inoculated with the 40% sucrose treated ascites cells thus providing additional evidence of viability.

Application of Schreck's staining test(6) to a sample of 40% sucrose-treated cells showed that at least 50% were definitely viable.

Discussion. Centrifugation of mixed ascites cells through a sucrose gradient appears to be a satisfactory method of obtaining pure

† Qo_2 values expressed as ul O_2/mg dry wt/hour. Respiration remained undiminished in measurements of Qo_2 over 60 min.

ascites tumor cells which are viable and morphologically intact though somewhat dehydrated. Since the fractionation achieved depends upon density of the various cells present as well as viscosity and temperature of the sucrose gradient, minor variations in degree of separation may be expected with samples of ascites fluid withdrawn at different times after inoculation of a host mouse. The method affords a rough fractionation between small and large ascites tumor cells, as well as between these and the erythrocytes and lymphocytes usually present in ascites fluid. The technic forms a useful basis for study of tumor cell-lymphocyte inter-relationships(7). For example, the influence of the lymphocytic cells on *in vitro* growth of ascites tumor cells is being studied in these laboratories.

Summary. Mixed cell types present in Ehrlich ascites tumor fluid have been separated by centrifugation through a sucrose gradient. The method affords a fractionation into 4 distinct bands of cell types. One of these appears to consist of pure ascites tumor cells, although no special technics were applied to determine whether any large mononuclears are also present in this band. Ascites cells removed from the gradient appear to be intact. The cell membrane is somewhat crinated and ratio of cytoplasmic to nuclear volume reduced owing to a certain amount of shrinkage through dehydration. The cells have been shown to be viable after contact with hypertonic sucrose in the gradient. Their oxygen uptake is equivalent to that of fresh untreated ascites cells, and they are capable of producing tumors when inoculated into mice. The separation technic forms a useful basis for study of tumor cells—lymphocyte inter-relationships in ascites fluid.

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Method for Isolating Single Cells and Preparation of Clones from Human Bone Marrow Cultures.* (24235)

DOROTHY M. SCHENCK AND MERWIN MOSKOWITZ (Introduced by W. A. Hiestand)
Dept. of Biological Sciences, Purdue University, Lafayette, Ind.

In our studies with normal human bone marrow cells† attempts were made to obtain cultures derived from single cells. The technic of Sanford(1) using capillary tubes proved inefficient and the plating technic of Puck (2) did not appear reliable insofar as certainty that the clones were derived from a single cell. When these cells are plated in a Petri dish they grow in apparent clones on the glass; however, inasmuch as they migrate on surface of glass and are occasionally released from the glass and settle at other locations we could not be certain that a culture obtained from such a colony had indeed been derived from a single cell. To obtain cultures which would unquestionably be derived from single cells the technic which will be described was developed. This technic involves placing small glass squares in a Petri dish, adding a suspension of cells to the dish, removing the individual glass squares and observing them microscopically, selecting glass squares con-

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† The cells were the Mox strain obtained from Dr. McCulloch of Connaught Med. Res. Labs., Univ. of Toronto, Can. This is an established strain normally maintained on medium 1066 plus 20% horse serum. The 1066 is obtained from Connaught Med. Res. Labs.

taining a single cell and placing them in culture vessels.

Materials. Glass squares. The glass squares were of pyrex glass 3 mm square and 0.13-0.16 mm thick. They were at first cut from #1 Corning brand coverslips after coverslips had been cleaned by tissue culture methods. They were later obtained cut to specifications by the Corning Glass Works. The latter squares were more uniform in size and had smoother edges, but were somewhat more difficult to clean because of their size. The observation apparatus consists of a dust guard, sterile moist cotton pledgets, sterile glass slide, and slide carrier (Fig. 1.) The dust guard was made by cementing 2 pieces of small glass tubing (3 mm o.d.) to edges of a glass slide. The slide carrier consists of plastic platform 15 mm high and is used merely to raise the remainder of the apparatus so that it may be more easily handled. *Vial cultures.* Flat bottomed shell vials 15 x 45 mm were used as culture vessels. To permit microscopic examination and photography with an inverted microscope, glass caps were used instead of rubber stoppers. The caps, 20 mm in height, were made by cutting off 19 x 65 mm vials. *CO_2 chamber.* Vial cultures were placed in desiccator for incubation. To facilitate handling, a long bolt was attached to the porcelain plate. Glass caps similar to those mentioned above were cemented to the plate to hold the culture vials. A small amount of water was placed in bottom of desiccator to minimize evaporation of fluid from the cultures. The pH of cultures was adjusted with CO_2. *Procedure.* One ml of nutrient medium was added to 60 mm Petri dish containing 30-36 of the 3 mm glass squares. The Petri dish was then shaken until all glass squares were wet and sank to bottom. Cells were washed from glass surface of culture vessel by forceful pipetting with a curved capillary pipette or trypsinized off with trypsin solution (0.05% Difco 1/250 in medium 1066). A cell count was taken with a hemocytometer and the cells diluted with nutrient medium to final concentration of 150 cells/4 ml. The cell suspension was added to the Petri dish and gently rotated to disperse the cells. The Petri dish was placed in a desiccator and the pH adjusted to 7.2 with CO_2. The culture was incubated at 37°C until cells settled and adhered to the glass. Time varied from 2 to 20 hours depending on cell type. The Petri dish was then rotated gently and the fluid discarded to remove unattached cells. Five cc of new nutrient fluid were added. The small glass squares were then examined for presence of single, healthy-appearing cells by removing them and observing them under microscope with the apparatus shown in Fig. 1. A sterile glass slide (B) was placed on slide carrier (C). Five or 6 of the glass squares were picked up with fine forceps and placed on glass slide. Care must be taken that the drop of fluid accompanying each square is spread on the glass so that no dark areas at edge of drop are present. A moistened, sterile cotton pledget was placed at each end of the slide to decrease evaporation of nutrient fluid and the slide was covered with a sterile dust guard (A). The glass squares were scanned at 75X magnification. If one cell was observed on a glass square the glass square and drop of fluid were reexamined at a higher magnification to verify presence of but a single cell. The desired glass squares were then transferred to vials containing 0.6 ml of medium preheated to 37°C. The vials were placed in the desiccator and

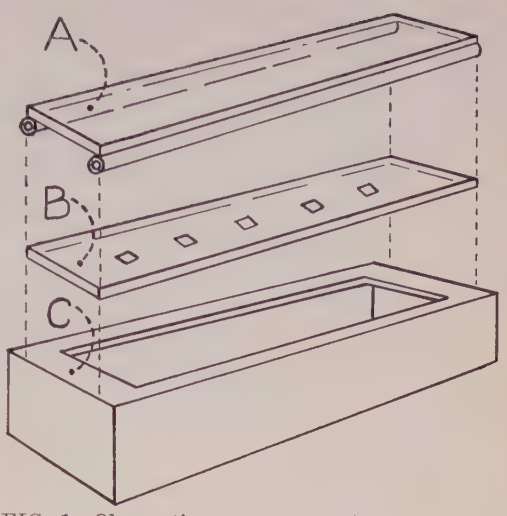

FIG. 1. Observation apparatus. A: Dust guard. B: Glass slide containing glass squares. C: Slide carrier.

pH adjusted with CO_2. They were incubated at 37°C until a relatively large colony developed. The colony was broken up and the cells dispersed over bottom of vial by forceful pipetting with capillary pipette. The cultures were reincubated until growth covered the bottom of vial. At that time the cells were loosened from the glass by forceful pipetting with a capillary pipette and the cell suspension transferred to a 13 x 100 mm pyrex tube. Fresh nutrient fluid was added to the vial and this culture was maintained until the clone was well established in tubes.

In some instances the cells were grown in solid medium. Three-tenths ml of medium containing 0.4% agar was placed in the vial and allowed to harden, the glass square was dropped onto the surface, and 0.3 ml of agar was then added on top. A colony developing in agar was allowed to grow until macroscopic in size. It was then dissected out, transferred to a vial containing 0.6 ml of liquid medium and thereafter treated as above.

Discussion. The percentage of glass squares found to contain but a single cell ranged from 12 to 27% of those examined. The percentage of single cells on glass squares that developed into cultures under specific conditions ranged from zero to 70%. Under a specific set of conditions some cells have proliferated well, whereas others have failed to proliferate or have done so very slowly, developing into transferable colonies only after 78 days as compared to 22 days for the former. All the different types of cells isolated from the Mox strain, at least those that can be divided into types on the basis of their morphology, are capable of developing into cultures from single cells but will do so only when growth conditions are appropriate. For instance, with one type of cell no colonies developed from single cells in the regular medium, but 60% of the cells proliferated well when the serum concentration was decreased.

This technic has certain advantages. Since the cell is attached to glass rather than suspended in fluid its morphology and condition may be more easily determined. Only cells which appear to be healthy need be selected for use. In instances where cells may be differentiated on morphological grounds, the selection of a particular type of cell is possible. The small glass squares are easily handled and may be transferred with a minimum of mechanical trauma to the cell to any desired culture vessel or to testing apparatus if individual cells are to be studied. When a particular type of cell does not grow under the regular cultural conditions it is possible to alter the conditions under which attempts are made to grow the single cells. By using glass vials with glass caps rather than tubes for culturing the cells, the depth of fluid may be varied easily without interfering with microscopic examination.

A disadvantage of this technic is that in the course of observing the cells on the glass squares they are exposed to light which may injure them. This, however, is an unavoidable evil with any procedure for isolating single cells in which microscopic examination is necessary. The change in oxidation-reduction potential in the course of microscopic examination may also affect the cells, but if the examination is done rapidly these effects can be minimized. There is the possibility of a second small cell being attached to the edge of the glass square where it cannot easily be detected. However, if this were the case, more than one colony would be detected growing and in no case has this been found to occur.

Although the technic has been utilized thus far only with bone marrow cells it would appear to be applicable for use with other types of cells. The conditions under which clone cultures may be developed from different types of cells may vary, but with the cells attached to the glass squares it is a simple matter to introduce the glass square into the necessary cultural conditions. Although a particular type of cell in a mixed culture may be maintained over many passages in association with other types of cells under a particular set of conditions, it may not be able to grow under such conditions by itself. With this technic it is possible to select cells that appear similar and attempt to grow them under a variety of cultural conditions to determine those which are optimum or necessary for the multiplication of the single cell. It

may be necessary to alter the nutrient medium, depth of fluid, gaseous environment, pH, or temperature of incubation in order to get a particular type of cell to grow. The nutrient medium may be pre-conditioned by previous growth of living cells or an irradiated feeder layer(2) may be used.

In attempting to establish clones from primary isolates of tissue, simply plating cells out involves an initial selection process in that only those types of cells that can grow under the conditions used will do so. However, with this technic, a partciular type of cell may be selected and placed under a variety of different cultural conditions in the hope of finding one that will permit the growth of the desired type of cell. Attempts are currently under way to isolate single cells directly from bone marrow and to develop from them clone cultures which can be maintained in serial passage.

Summary. A simple method for isolating single tissue culture cells has been developed. The cells are isolated on small glass squares which can be transferred to any desired culture vessel or testing apparatus. This procedure has been used for the preparation of clone cultures.

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Immediate Effects of Intravenous Injections of Tolbutamide and Insulin on Blood Glucose and Amino Acids.* (24236)

ROGER C. DEMEUTTER, AVEDIS K. KHACHADURIAN AND ALEXANDER MARBLE
Cancer Research Institute and Baker Clinic Research Laboratory, N. E. Deaconess Hospital, Boston, Mass.

After several years of study there is no general agreement regarding the details of the mechanism of action of the hypoglycemic sulfonylureas and more studies are needed to clarify this problem(1,2). The present study was undertaken with two purposes in mind: (1) To determine the time of onset of hypoglycemic action of tolbutamide following its intravenous administration and to compare, in normal and diabetic individuals, the blood glucose curve thus obtained with that produced by insulin; (2) To determine if, like insulin, tolbutamide causes a fall in the serum amino acids.

Materials and methods. Three groups of individuals were studied: (1) Five normal male volunteers, age 28 to 35, body weight 70 to 80 kg. (2) Seven patients with stable diabetes, age 42 to 73, weight 70 to 88 kg, classified as suitable for tolbutamide therapy according to commonly accepted criteria(3). (3) Two patients with unstable diabetes requiring more than 50 units of insulin daily. Prior to study, all subjects were fasted 12 hours. Patients on long-acting insulin were taken off this preparation for more than 48 hours and, when necessary, crystalline insulin was given for control of diabetes. However, no insulin was given later than 15 hours prior to test. The tests were performed on recumbent, rested subjects; no smoking was allowed. Blood samples were drawn through a needle fitted with stylet and kept in place in a brachial vein during the whole test. Stasis was avoided and no tourniquet used whenever possible. After a short control period of saline infusion, glucagon-free insulin† was injected intravenously at fixed dose of 0.1 unit/kg actual net body weight. One or several days later the same procedure was used and sodium

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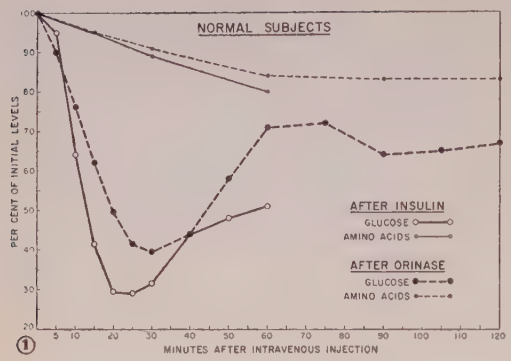

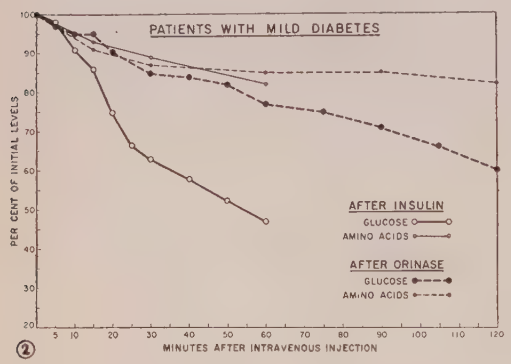

FIG. 1 (top). Response of blood glucose and serum total alpha amino acids following intrav. inj. of glucagon-free insulin (0.1 unit/kg body wt) and sodium tolbutamide (Orinase) (40 mg/kg) to 5 normal male human subjects. Points shown in the curves represent % of levels prior to injections.

FIG. 2 (bottom). Response of blood glucose and serum total alpha amino acids following intrav. inj. of glucogan-free insulin and sodium tolbutamide (Orinase) in 7 patients with stable diabetes. Doses of insulin and of sodium tolbutamide were as given in Fig. 1. Points shown in the curves represent % of levels prior to injections.

tolbutamide in the same dosage of 40 mg/kg was injected intravenously in the form of 10% solution in saline with pH 9.3. During its rapid injection (3 minutes) most subjects experienced a slight burning along the vein. Blood glucose determinations were made according to the Somogyi-Nelson method(4). Serum total alpha-amino acids were determined by the ninhydrin colorimetric method (5) using tungstic acid as the deproteinizing agent.

Results. Blood glucose. Fig. 1 and 2 represent mean values for blood glucose expressed as per cent of fasting level. In normal subjects, tolbutamide produced a rapid decrease in blood glucose which began within 5 minutes in 3 of 5 subjects. The maximum fall with insulin occurred at 25 minutes and was 71% of the fasting value, while with tolbutamide it occurred at 30 minutes and was 61% of the fasting value.

In 7 patients with stable diabetes decrease in blood glucose with insulin was slower and smaller than in the normal group, averaging 56% at 60 minutes (end of experiment). However, the response to tolbutamide was even less than that due to insulin; a mean maximum fall of 40% was attained at the end of experiment at 120 minutes.

In the 2 non-responsive diabetics there was no drop in blood sugar after tolbutamide while one of them who received insulin showed a fall of 34% at 2 hours.

Serum amino acids. There was no difference in fasting levels of normal and diabetic subjects. In normal patients with stable diabetes, tolbutamide caused a decrease in serum amino acids comparable to that produced by insulin. At 60 minutes the normal subjects had a fall of 20% with insulin and 16% with tolbutamide, while the responsive diabetics had a fall of 18 and 15% respectively. Following tolbutamide there was no decrease in the amino acids of the 2 patients with unstable diabetes, while a 20% fall occurred after insulin in one of these patients. The onset of the fall in amino acids was comparable in the different experiments and a significant fall occurred in the majority of patients at 15 minutes.

Discussion. Our results with the effect on blood glucose of tolbutamide agree with those of other workers(1) and, moreover, they indicate that onset of action of tolbutamide is almost immediate since a significant fall in blood glucose occurs within 5 minutes after its injection.

If tolbutamide acts by increasing insulin output from the pancreas, it would appear to do so, at least initially, by releasing preformed insulin. The fact that the pancreas of the diabetic contains less extractable insulin than that of the normal, fits with the observation that the hypoglycemic effect of tolbutamide is less marked in the diabetic subjects. The fall observed in amino acids after tolbutamide is

consistent with the observations of Recant and Fischer(6) that pretreatment of rats with tolbutamide causes a greater incorporation of glycine into protein by their livers. Therefore, as determined by these 2 studies, tolbutamide when effective in producing hypoglycemia, has an action parallel to that of insulin on amino acid metabolism(7). This similarity of action adds support to the evidence that tolbutamide acts by increasing the output of insulin and speaks against the possibility that depression of gluconeogenesis from amino acids could be a factor in production of hypoglycemia.

Summary. 1) In normal subjects sodium tolbutamide given intravenously in dosage of 40 mg/kg of actual body weight produced hypoglycemia comparable to that produced by insulin (0.1 unit/kg body weight) intravenously. In 3 of 5 subjects the fall in blood glucose took place within 5 minutes following injection of tolbutamide. 2) In 7 patients with stable diabetes the decrease in blood glucose following tolbutamide was slower than that with insulin and no fall occurred in 2 patients with unstable diabetes. 3) In normal persons and in the 7 diabetic patients who experienced a fall in blood glucose following tolbutamide, there was likewise a decrease in serum amino acids comparable to that following insulin. No fall in serum amino acids occurred in the 2 patients with unstable diabetes.

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Pituitary and Plasma Bioassay for Trophic Hormones in the Alloxan-Diabetic Rat.* (24237)

A. M. LAWRENCE,† A. N. CONTOPOULOS AND M. E. SIMPSON

Depatrment of Anatomy and Inst. of Experimental Biology, University of California, Berkeley

Among endocrine disturbances reported in experimentally induced diabetes mellitus have been impairment of reproductive physiology and hypoactivity of the thyroid(1-6). In the present study the gonadotrophic, thyrotrophic, and growth-promoting potencies of the pituitary glands and blood plasma of diabetic rats were determined by bioassay in hypophysectomized immature female rats.

Materials and methods. Female rats of the Long-Evans strain maintained on an adequate natural food diet‡ were injected with alloxan at 50 days of age and sacrificed at 100 days of age. Pituitaries and plasma were assayed in hypophysectomized rats, 28 days old at operation, used 14 days later. Hypophysectomy was performed by the parapharyngeal approach. Completeness of ablation was checked by observation of the sella turcica at autopsy. Atrophy of target organs furnished

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‡ All diabetic rats were maintained on a modified McCollum Diet I consisting of ground whole wheat 67.5%, casein 15%, skim milk powder 7.5%, sodium chloride iodized 0.75%, calcium carbonate 1.5%, melted fat 6.75%, fish oil (Vit. A and D concentrate) 1%. KI solution added to 1 μg of iodine/g of diet (450 mg KI/liter, 300 cm^3/270.5 lb of diet). Hypophysectomized rats were offered daily a wet mash of this diet. All rats received a supplement of lettuce 2-3 times a week and were maintained at 74° ± 1°F temperature.

confirmatory evidence. To induce diabetes a 5% solution of alloxan (recrystallized) in isotonic saline was made immediately prior to injection. Prior to alloxan administration, the rats were fasted for a period not exceeding 72 hours. A dose of 5 mg/100 g body weight was injected rapidly into the tail vein. Access to food was allowed immediately after injection. The diabetic status was allowed to stabilize for 7 weeks and rats with blood sugar levels greater than 350 mg% were used as donors (controls, 112 mg %). Blood glucose levels were determined by Nelson's modification of the Somogyi procedure(7). Assays of plasma from diabetic and control rats for presence of trophic hormones were performed as follows. Whole blood from etherized donors was collected from the abdominal aorta into an heparinized syringe. Blood was quickly transferred to a conical centrifuge tube, covered with a layer of mineral oil, and centrifuged 12 minutes at 3000 rpm. The plasma was then aspirated into a syringe and injected into recipient hypophysectomized rats within less than 30 minutes after withdrawal. Recipient hypophysectomized animals received 4 daily injections of plasma. Assays of pituitaries of donor rats for trophic hormone content was conducted as follows. Pituitaries were dissected immediately after exsanguination, posterior and intermediate lobes were separated, and anterior lobes were quickly frozen on dry ice. Suspensions for injection were prepared immediately before the assay period. The glands were weighed and ground by motor-driven ground glass rod inside a test tube, known amounts of isotonic saline being added. Butanol (1%) was added and suspensions were stored at 0-4°C during the 4-day injection period. Recipients were autopsied 24 hours after the last injection. Ovaries and uteri with oviducts were weighed, fixed in Bouin's solution, sectioned and stained. The tibias were split, fixed in 10% formalin, and stained with silver nitrate. Eighteen hours prior to autopsy all assay animals received intraperitoneally a tracer dose of I^{131} (0.5-1 μc). Thyroids were placed in fixative in a small vial suitable for counting radioactivity(8). Differences between counts/minute of thyroids and of a leg muscle fragment of comparable size, expressed as percent of injected dose, represented the I^{131} uptake by thyroid gland. Gonadotrophic potency of pituitaries and plasma was determined by microscopic structure of ovaries of recipients (9, 10).§ Thyrotrophic hormone content of pituitary glands and plasma was appraised on the basis of iodine-concentrating capacity and histology of thyroids from assay animals. An I^{131} uptake greater than 1% of injected radioactivity has been adopted as indicating minimal thyrotrophic stimulus. The cytological criteria adopted for determining minimal effective dose for thyrotrophic stimulation were an increase in height of follicular cells to low cuboidal, associated with some loosening of nuclear chromatin and beginning vacuolation of colloid. Evaluation of growth-stimulating activity was based on ocular-micrometer measurements of the uncalcified proximal epiphyseal cartilage of the tibia(11). A width increment of 50 μ above hypophysectomized control level, or an absolute value of 200 μ was regarded as a significant response. Bioassays were repeated at least once for each dose level.

Results. Bioassay of anterior pituitary hormone content. Dose levels between 1/32 gland and 4 anterior lobes from diabetic rats were assayed in hypophysectomized recipients (Table I). A total dose of 2 glands was required to increase ovarian weight; a dose of 4 glands also increased uterine weight. Minimal stimulation to follicular growth resulted

§ Minimum effective dose for follicle stimulating hormone was judged by appearance of healthy small-medium follicles with beginning antrum formation. Degree of follicular growth was determined by measurement of diameter of health (non-atretic) follicles by the aid of an ocular micrometer. The following gauge is used throughout this study: small follicles, sF = 0.375 mm; small-medium follicles, smF = 0.375-0.5 mm; medium follicles, mF = 0.5-0.65 mm; medium-large follicles, mlF = 0.65-0.75 mm; large follicles lF = 0.75-1.0 mm. Minimum effective dose for interstitial-cell-stimulating hormone was judged by beginning repair of ovarian interstitial tissue. This was noted by an increase in vesicularity of the nuclear pattern, accompanied by an increase in size and depth of staining of these cells.

TABLE I. Gonadotropic, Thyrotrophic and Growth Hormone Potency of Anterior Pituitaries of Adult Female Diabetic Rats as Compared With Normal. (Assayed in hypophysectomized rats, 8 rats/group.)

Type of donors	No. of rats	Total dose: No. of glands	Total dose: Wt, mg	Uterus wt, mg	Ovaries: Wt, mg	Ovaries: Histology: Follicles	Ovaries: Histology: Interstitial cells	Thyroid: I^{131} uptake, %	Thyroid: Histology	Tibial cartilage width, μ
Diabetic	21	0	0	24 ± 2	9 ± 1	sF	Atrophic	.50 ± .1	—	154 ± 2
	8	1/32	.15					.92 ± .1	—	153 ± 4
	7	1/16	.3					1.25 ± .1	+ (MED)	159 ± 3
	8	1/8	.6					2.34 ± .2	+	164 ± 3
	8	1/4	1.1					3.07 ± .3	+	161 ± 3
	8	1/2	2.2	26 ± 3	10 ± 2	sF	Atrophic	4.52 ± .5	+	191 ± 2
	8	1	4.5	24 ± 2	10 ± 1	sF	Partial repair (MED)	5.68 ± .9	+ +	203 ± 4 (MED)
Normal	8	2	9.0	30 ± 3	17 ± 1	s, smF (MED)	Repair	9.61 ± 2.5	+ +	245 ± 5
	8	4	18.0	98 ± 2	39 ± 7	sm, m, 1F, CL	"	16.68 ± 1.8	+ + (+ + +)	263 ± 6
	15	1/32	.3					.95 ± .2	—	193 ± 4
	22	1/16	.6					1.53 ± .3	+ (MED)	202 ± 5 (MED)
	22	1/8	1.2					2.62 ± .5	+	232 ± 5
	12	1/4	2.4					3.94 ± .6	+ +	248 ± 4
	16	1/2	4.8	24 ± 2	9 ± 1	sF	Atrophic	5.72 ± .4	+ +	286 ± 5
	14	1	9.7	27 ± 1	11 ± 1	sF	Repair (MED)	7.85 ± 1.1	+ +	297 ± 8
	12	2	19.4	35 ± 4	15 ± 2	s, smF (MED)	Repair	10.95 ± 1.5	+ + (+ + +)	308 ± 8
	6	4	38.8	124 ± 4	35 ± 3	sm, m, 1F, CL	"	18.50 ± 1.7	+ + +	316 ± 12

after administration of the 2 glands (9 mg) and corpora lutea were present after 4 glands. Beginning repair of the interstitial tissue was detectable at the 1 gland level (4.5 mg). The minimal effective doses did not therefore differ from those of pituitaries of normal female rats of the same age (Table I)(12). In this connection attention is called to the disparity between pituitary weights in diabetic and normal rats; glands from diabetic rats were approximately half normal weight, 4.5 mg compared with 9.7 mg. The body weight of diabetic rats was also half normal, 146 g compared with 233 g so that pituitary weight remained the same percent of body weight. A dose of 1/16 pituitary gland from diabetic rats elicited a minimum response in the thyroid of hypophysectomized recipients, judged both by histology of the thyroid and I^{131} uptake. Comparison with assay of normal pituitary glands, shows that 1/16 anterior lobe was also minimal for thyrotrophic hormone activity (Table I). A dose of 1 anterior pituitary from diabetic rats caused a significant increase in width of the epiphyseal plate. Pituitares of normal female rats of the same age gave minimal growth stimulation at 1/16 of an anterior lobe. The growth hormone content of anterior pituitary glands from diabetic female rats was therefore markedly reduced (Table I).

Bioassay of blood plasma. Table II shows that growth-promoting potency of blood serum was reduced in alloxan-induced diabetic female rats, as compared with normal rat plasma. At a daily dose of 3.5 to 4 cc the plasma of normal donors gave a significant increase in tibial epiphyseal width(13), where-

TABLE II. Decreased Growth Promoting Potency of Blood Plasma of Diabetic Rats.

Donor group	Daily dose, ml	No. of rats per assay	Body wt, g: Initial	Body wt, g: Final	Width of proximal epiphyseal cartilage of tibia, μ
Diabetic	4.0	4	66	68	190 ± 2
	3.5	3	70	75	186 ± 4
Normal	4.0	4	67	73	210 ± 9
	3.5	4	66	72	214 ± 5
Uninj. recipients	0.0	4	64	68	169 ± 3

as plasma of diabetic donors gave only a marginal response. No other hormones were detected in plasma of diabetic and normal rats at doses tested.

Discussion. Houssay and Foglia reported a decrease in gonadotrophic content in the pituitaries of alloxan injected diabetic rats (14), but no other differences were detected between trophic hormone potencies of pituitaries of diabetic and normal rats. Diabetic rats used in this study were 7 weeks post-alloxan treatment, whereas in studies of Houssay and Foglia the rats were sacrificed 48 hours after administration of alloxan. In the present study no significant differences were found between diabetic and normal adult female pituitary gonadotrophic and thyrotrophic potency whereas a 16 fold reduction in growth hormone content was found in pituitaries from diabetic female rats. Growth hormone potency of pituitaries was reduced, whether measured in fractions of anterior lobe, or in mg of pituitary tissue. There was also a reduction in growth-promoting activity of plasma of diabetic rats, as judged by tibial cartilage width.

Pituitary trophic hormone content should always be considered in relation to hormone production and release. The similarity in gonadotrophic and thyrotrophic content of pituitaries from normal and diabetic rats does not necessarily indicate production of these hormones at the same rate. Differences in secretion rate and storage might result in the same final content. The atrophic status of the gonads observed in diabetic donors(1-4) indicates reduced secretion of gonadotrophic hormones, unless the response of these target organs to the trophic hormones is different in diabetic animals. The reduced growth promoting potency of plasma can not be attributed solely to decreased growth hormone release from the pituitary without consideration of changes of titers of other hormones.

Summary. The gonadotrophic, thyrotrophic, and growth hormone content of anterior pituitaries from female rats rendered diabetic by alloxan was determined by bioassay in hypophysectomized female rats. Trophic hormones in blood plasma were also determined. The number of glands required to evoke minimum follicle stimulating, interstitial cell stimulating, and thyrotrophic activity were the same in diabetic and normal rats, whereas growth hormone content of the pituitary was reduced 16-fold. The titer of circulating growth promoting factors was also reduced.

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Intraperitoneal Insemination of Rabbit Doe.* (24238)

R. Hadek (Introduced by Earl A. Hewitt)
Department of Veterinary Anatomy, Iowa State College, Ames, Ia.

While trying to develop a suitable technic for artificial insemination of rabbits, the intraperitoneal route offered itself as a possibility. The rabbit is a conveniently suited animal for this type of work, since ovulation takes place 10 hours after insemination(5). To check feasibility of the technic some rabbits were administered one cc India ink into the peritoneal cavity. This mostly disappeared after one hour and substantial amounts were recovered in the oviducts.

Technics. In this pilot study started in 1957, doe rabbits were mated to vasectomised bucks and 1 to 12 hours after mating they were inseminated through the linea alba with freshly ejaculated rabbit semen. The semen was collected from suitably trained bucks with the help of an artificial vagina, which in turn is a modification of Macirone-Walton's (6) old model. The semen, after having been tested for motility, was either injected immediately, or was treated in one of 3 different ways. (1) Diluted to double its amount, (2) centrifuged, the supernatant fluid discarded and the semen resuspended in 1 cc sodium citrate buffer at pH 7.4, or (3) the resuspended semen was centrifuged again and after discarding this supernatant, newly resuspended in 1 cc citrate buffer (2x washed). Only semen which had shown good motility was used. The procedure adopted was: To suspend females by their hind legs and inject the semen approximately 5 inches cranial to pelvic inlet through the linea alba. A 2-inch 14 gage hypodermic needle was used. To evaluate the result, the females were sacrificed one week to 10 days after insemination. This abdominal insemination has been performed on 30 animals.

Results. No fertilization occurred in animals which were inseminated with once washed sperms. Blastocyst formation was observed in animals which were inseminated with twice washed sperms. However, all blastocysts which were obtained from injections later than 2 hours after mating were degenerating, while those obtained as a result of insemination within 2 hours after mating were viable (Table I). These results suggest 2 important factors which influence success of intraperitoneal insemination. (1) Time elapse between mating (ovulation) and insemination, and (2) suspending media of the semen.

TABLE I. Results of Fertilization Experiments.

Exp.	No. of rabbits	Treatment of semen	Time of inj. after mating (hr)	Avg No. of blastocysts	Outcome of exp.
A	4	None	8	0	—
B	3	Diluent	8	0	—
C	6	1× washed	8	0	—
D	6	2× "	8	.5	±
E	4	*Idem*	6	2	±
F	3	"	4	2.5	±
G	4	"	1–2	6	+

—, no ovum fertilized; ±, blastocyst development stopped at very early stage; +, viable blastocysts developed.

With regard to (1), optimal result was obtained in those animals which were injected 1-2 hours after mating—8 hours before ovulation. As far as (2) is concerned, the presence of accessory genital gland secretion suggests an interference with fertilization of the ovum of the rabbit. No blastocysts were formed when accessory genital gland secretion was present.

Discussion. The experiment shows that intraperitoneal insemination in the rabbit is a possibility. Since it is more successful than vaginal insemination(3) and less cumbersome than complete laparotomy, to deposit semen in the uterus(4), it offers a few advantages.

If the present studies shed any new light on the "capacitation factor" of Austin(1) and Chang(2), it is that capacitation of intraperitoneally injected semen takes longer than those which were deposited in uterus or oviduct(2,4).

* This study was partly aided by Ortho Research Grant awarded by Am. Soc. for Study of Sterility.

While this work was in progress, 2 papers appeared whose authors proved that intraperitoneal insemination is feasible in the bovine(7), although the results were not encouraging, and in the guinea pig(8), where only epididymal semen was used. Further work on this problem is still in progress.

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Platelets XXI. Glutamic Pyruvic Transaminase Activity of Human Platelets.* (24239)

GEORGE TSITOURIS† AND MARIO STEFANINI

Joseph Stanton Memorial Laboratories, Saint Elizabeth's Hospital and Department of Medicine, Tufts University Medical School, Boston, Mass.

Previous work has shown that human blood platelets represent a source of glutamic oxalacetic transaminase (GO-T)(1). Observations have now been extended to the related enzyme, glutamic pyruvic transaminase (GP-T). The study appears justified since both enzymes have been found in serum and in homogenates of many tissues with wide differences of activity from tissue to tissue and from health to disease.

Materials and methods. The activity of GO-T and GP-T was investigated in serum, platelet-rich and platelet-poor plasma, washed platelets, washings of isolated platelets and water soluble platelet extracts. All materials were of human source. Serum was obtained by centrifugation from clotted blood, collected in sterile syringes and incubated at 37° for one hour. To prepare platelet-rich and platelet-poor plasma, blood was collected in Silicone-coated test tubes, using Sequestrene as anticoagulant(2). To obtain platelet-rich plasma, the blood was centrifuged at 1,000 rpm/10′/4°C; and at 3,000 rpm/30′/4°C for platelet-poor plasma. The upper two-thirds of supernatant plasma were transferred to fresh Silicone-coated test tubes. Platelets were prepared by the technic previously described(2). They were washed 3 times with sterile isotonic saline solution using, each time, a volume identical to that of the platelet-rich plasma originally used. A discrete suspension was obtained, the platelets counted by the method previously described(3) using phase contrast microscope and their number adjusted to various levels by adding sufficient isotonic saline to the original platelet suspension. Water soluble platelet extracts were prepared exactly as described(1). Both GO-T and GP-T activity were determined within 2 hours of preparation of the reagents. The spectrophotometric technic of Karmen *et al.* (4) was used for determination of GO-T and that of Wróblewski and LaDue(5) for determination of GP-T. A minor modification was introduced since 0.03 ml of malic acid reagent was used rather than 0.1 ml as suggested in the original technics. All experiments were done in triplicate. The reacting mixture was incubated for 10 minutes and readings taken at 5, 10 and 15 minutes after adding alpha-ketoglutaric acid reagent. Therefore, the final figures reported represent the average of 9 values obtained for each sample. Duplicate determinations were also done using the colorimetric method of Reitman and Frankel(6).

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† Former Assistant in the Dept. of Medicine, University of Athens, Greece.

TABLE I. Glutamic Oxalacetic and Glutamic Pyruvic Transaminase Activity in Serum, Plasma and Various Platelet Preparations.*

Preparation	No. of platelets per mm³ (10^3)	GO-T activity, units/ml	GP-T activity, units/ml
Serum	—	47.6 ± 4.9	40.6 ± 5.8
Platelet-rich plasma	356 ± 12.3	46.3 ± 6.1	37.3 ± 6.3
" -poor "	13 ± 1.4	25.3 ± 3.2	36.2 ± 4.7
" suspensions	2,000 ±240	265 ±15.3	<1
	480 ± 27	59.3 ± 4.0	
	190 ± 19	47.2 ± 2.7	
	50 ± 3	29.4 ± 1.3	
Washings of sedimented platelets		12.5 ± .9	8.3 ± .9
Water soluble platelet fraction		109.7 ± 9.9	3.2 ± .3

* Avg of 5 experiments and stand. dev.

This technic appeared adequate, although less sensitive than the spectrophotometric procedure.

Results. (Table I). The figures obtained for GO-T activity in serum and platelets agreed closely with those previously reported (1). The serum GP-T activity was within the ranges reported by previous investigators. There was however an insignificant difference in GP-T activity between platelet-poor and platelet-rich plasma. Also platelet suspensions and water soluble platelet extracts contained negligible GP-T activity. These results suggested that human platelets contain no GP-T activity. Since, however, slight GP-T activity was detected in washings of sedimented platelets, it is possible that minute amounts of enzymatic activity are absorbed from serum on platelets. Two patients with acute infectious hepatitis and one with myocardial infarction were studied since serum GP-T activity is higher in these diseases. GO-T activity was greatly increased in serum, plasma and various platelet preparations. As expected, GP-T activity was very high in serum, platelet-poor and platelet-rich plasma, but only traces were present in platelet preparations and in water soluble platelet extracts (Table II).

To eliminate the presence of an inhibitor of GP-T activity in human platelets, equal volumes (0.1 ml) of untreated human serum and of a suspension of homologous platelets in isotonic saline (containing 190,000 platelets/cu mm) were incubated at 37°C for 3 hours. Also, in other experiments 100 mg of pyridoxal phosphate were added to the reacting mixture, since it has been shown that this reagent is a coenzyme of transaminases(7). Because of the possibility that platelets may not be permeable to the added coenzyme or that an inhibitor may not diffuse out of intact platelets these experiments were repeated using water soluble platelet extracts. There was no change in GP-T activity of serum beyond that accounted for by dilution.

Discussion. It appears that human platelets contain considerable GO-T but very little or no GP-T activity. The very low values of GP-T detected may even indicate that washing of platelets was insufficient to remove contaminating plasma. Whether this dissocia-

TABLE II. Comparative Glutamic Oxalacetic and Glutamic Pyruvic Transaminase Activity in Serum, Plasma and Platelet Suspensions in 3 Patients.

	Infectious hepatitis		Infectious hepatitis		Acute myocardial infarction	
	GO-T	GP-T	GO-T	GP-T	GO-T	GP-T
	Units/ml					
Serum	245	500	220	390	184	142
Platelet-rich plasma	240	475	210	382	178	136
" -poor "	182	475	137	378	132	135
" suspensions (200,000 platelets/mm³)	72	10	45	11	62	2

tion, which has been observed in many tissues as well(8), has any significance, especially with reference to the various physiologic functions of platelets, remains to be further investigated.

Summary. (1) Platelets are a source of glutamic oxalacetic transaminase in human blood. (2) Platelets, however, contain only traces of glutamic pyruvic transaminase in normal and pathologic conditions. This finding is not explained by presence of enzyme inhibitors or by lack of a co-enzyme. The significance of the dissociation between GO-T and GP-T activity in platelets, as for other tissues, remains obscure.

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A Method for Assaying for Glycoprotein in Column Fractionation. (24240)

BERNARD RABINOVITCH AND RICHARD BARTOSIEWICZ
Argonne Cancer Research Hospital, USAEC, and Department of Biochemistry, University of Chicago

In the course of experiments involving chromatographic and electrophoretic separation of proteins on a column with subsequent collection of as many as 400 tubes containing eluate from the column, we have been faced with the task of assaying each of these tubes for glycoprotein. Several methods have been described(1-4) for determining qualitatively, semi-quantitatively, and quantitatively carbohydrate; but none can be used conveniently for repeated routine assay purposes. We adapted a staining technic for carbohydrate, which can be managed with a minimum of practice and a maximum of convenience and which will give quantitative determinations with an estimated accuracy of 5%.

Method. The method involves the use of Köiw's stain devised for use with paper electrophoretic or chromatographic strips. The technic for its use is described elsewhere(5). Aliquot samples from each tube are withdrawn and applied to strip of paper by means of a striper supplied with the Spinco hanging-strip paper electrophoresis apparatus. Samples of 10 λ may be applied in a single application, but larger samples are best applied in multiple applications. The paper strip should be that supplied in the Spinco paper electrophoresis apparatus. The samples should be applied about ¼ inch apart at prelocated points on the strip marked by pencilled dots, starting about 3½ inches from one end. About 25-30 samples may be applied to one strip.

Application of sample to the paper is critical inasmuch as there will be an accumulation of carbohydrate at edges of the band formed by the sample, if it is left to dry in air at room temperature after application. Should this happen, quantitative determination is impossible. We placed the paper strip on a slide warmer at temperature about 80°C, applying the samples to the strips, and immediately thereafter passing a flow of warm air over them from a hair dryer. In this way, the sample is dry within 10 seconds. As many as 8 such strips may be stained simultaneously with Köiw's stain, and the resultant strips, after drying, should be passed immediately through the Spinco Analytrol for a permanent record and quantitative determination. If

necessary, a known amount of glycoprotein or other standard may be applied to each strip by way of calibration reference. Integration under the curves obtained on the Analytrol is readily performed, but the height of peaks may be used instead for quantitative determination. The Analytrol should be made to scan a sufficient distance along the strip where no samples have been applied, to obtain adequate determination of the background. It may be possible to obtain improved results by use of green light filters (*e.g.*, Klett 52) instead of standard blue filters found in the Analytrol.

Results. Fig. 1 shows the kind of results obtained by assaying for carbohydrate in the region of a peak eluted from a calcium phosphate column and detected by absorption at 280 mμ. The material placed on the column was a bovine fraction VI and the eluent was a 0.06 M phosphate buffer at pH 6.8.

Fig. 2 shows results of applying this method to the location of a glycoprotein fraction in the column electrophoresis of bovine fraction VI to which had been added I^{131} labeled thyroxine. The LKB column electrophoresis apparatus packed with Solka Floc (cellulose), 200 mesh, was used. Two hundred mg of protein was placed on the column and electrophoresed at 5 ma and 770 volts for 29 hours in a barbital buffer medium at pH 8.6, 0.0125 M. The column was eluted at 60 seconds/drop with barbital buffer, and 1 ml fractions collected. It is worth noting that the glycoprotein peak coincides with the radioactivity peak but not exactly with the OD 280 mμ peak, and this result has been confirmed.

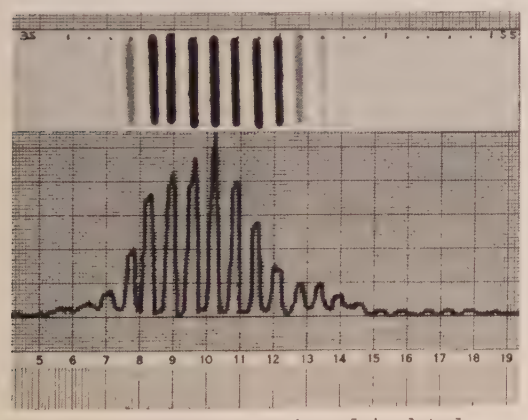

FIG. 1. Developed paper strip and Analytrol scan. Assay for glycoprotein in a chromatographic separation of fraction VI.

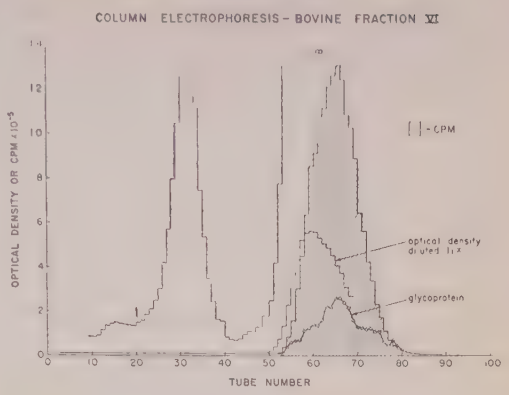

FIG. 2. Column electrophoresis of bovine fraction VI. Shaded area represents location of thyroxine.

Summary. A convenient method for qualitative or quantitative determination of carbohydrate in many samples has been worked out. The method involved the deposition of micro quantities of the samples on paper strips, staining with Köiw's stain and analysis of the results with the Spinco Analytrol apparatus. The method, applied to the fractions obtained from the column electrophoresis of bovine fraction VI on a cellulose column, demonstrated the presence of a glycoprotein component which binds I^{131} labeled thyroxine.

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Effect of Chlorpromazine on Antidiuretic Response to Noxious Stimuli.* (24241)

David de Wied and Robert Jinks (Introduced by I. Arthur Mirsky)
Department of Clinical Science, University of Pittsburgh School of Medicine

Exposure of animal or man to noxious stimuli results in an antidiuretic response(1,2,3) with which is associated an increase in the antidiuretic activity of the plasma(3,4,5). Since administration of chlorpromazine reduces the anxiety provoking effect of various environmental stimuli(6,7), it became of interest to examine the effect of such treatment on antidiuretic response to different types of noxious stimuli.

Materials and methods. Male albino rats of the Carworth strain, weighing from 120-200 g, were used after a preliminary fast of approximately 16 hours during which time the animals were permitted access to water. During the experiment the rats were kept in individual metabolic cages. The animals were given 5 ml 0.2% NaCl per 100 g body weight by stomach tube and one hour later the gavage was repeated. At 20 minute intervals after the second gavage, volume of urine excreted was measured. All rates of excretion were expressed as ml/100 g of body weight. The animals were handled and prodded just prior to each measurement of urine volume in order to ensure the emptying of their bladders. The noxious stimulus was applied at 20 minutes after the second gavage.

Three types of noxious stimuli were used in this study. One group of rats was exposed to painful stimuli produced by a 2 minute period of mild electric shocks (50 volts, 60,000 ohms) to the feet *via* a grid which made up the floor of the metabolic cage. A second group of rats was given an intraperitoneal injection of 1 mg histamine dihydrochloride. A third group of rats was given an intraperitoneal injection of 0.1 mg nicotine-tartrate per 100 g body weight. For each experimental group another group of rats was used as controls in that the rats were not exposed to noxious stimuli; those animals which served as controls for the histamine and nicotine treated groups received an injection of an equivalent amount of normal saline. A similar number of groups of rats were treated the same way except that they were given a subcutaneous injection of 0.25 mg chlorpromazine per 100 g body weight 60 minutes before the first gavage.

Results. The rate of secretion of urine by the rats used as controls was fairly constant after the second gavage while the animals exposed to noxious stimuli developed an antidiuretic response (Fig. 1 A). Whereas the group which served as its control excreted 1.54 $\pm$ 0.08 ml urine per 100 g body weight during the second 20 minute interval, the group of rats which was exposed to painful stimuli excreted 0.88 $\pm$ 0.09 ml during the same interval. The difference between the 2 rates was statistically significant ($P<0.001$).

A more intense antidiuretic response followed administration of histamine and nicotine (Fig. 1 A). Thus, the animals treated with histamine excreted 0.48 $\pm$ 0.12 ml while their controls excreted 1.64 $\pm$ 0.11 ml during the 20-minute interval after injection ($P<0.001$). During the 20 to 40 minute interval after injection of histamine the rats excreted 0.39 $\pm$ 0.11 ml while their controls excreted 1.31 $\pm$ 0.10 ml. Likewise, during the same interval the nicotine-injected animals excreted 0.60 $\pm$ 0.13 ml while their controls excreted 1.60 $\pm$ 0.12 ml ($P<0.001$).

Administration of 0.25 mg chlorpromazine per 100 g body weight produced a statistically significant but transient antidiuretic response. Whereas the saline treated groups excreted 1.82 $\pm$ 0.13 ml during the first hour after the first gavage, the chlorpromazine treated rats excreted 1.35 $\pm$ 0.14 ml during the same interval ($P<0.02$). However, during the hour after the second gavage the saline treated rats excreted 4.3 $\pm$ 0.12 ml while the chlorproma-

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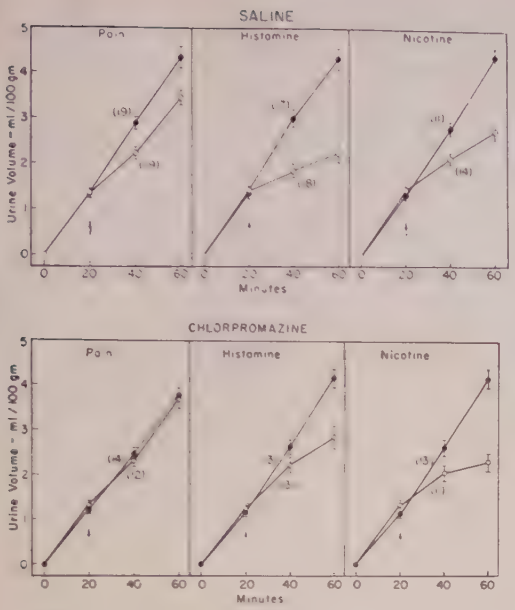

FIG. 1A (top). Antidiuretic response of saline treated rats to noxious stimuli.

FIG. 1B (bottom). Antidiuretic response of chlorpromazine treated rats to noxious stimuli. The mean ± SE ml excreted per 100 g of body wt of the groups that served as controls are designated by closed circles and that of the groups exposed to noxious stimuli by open circles. Numerals refer to No. of animals in each group. Arrows indicate time at which noxious stimulus was applied.

zine treated groups excreted 3.96 ± 0.14 ml. The difference between the two groups was statistically not significant. Accordingly, the antidiuretic effect of chlorpromazine appears to be an evanescent one.

Exposure to painful stimuli did not result in an antidiuretic response in the chlorpromazine treated rats (Fig. 1 B). Administration of histamine, however, produced a significant antidiuretic response which was most apparent during the third 20-minute interval after the gavage. Antidiuretic response to injection of nicotine (Fig. 1 B) was as marked as that observed in the saline-treated controls (Fig. 1 A).

Discussion. The dosage of chlorpromazine used in this study was twice that necessary for complete extinction of a conditioned avoidance response(6). Such dosages, however, have no apparent analgesic properties since the rats appeared to experience the pain as exhibited by their behavioral responses. Yet, pretreatment with chlorpromazine prevents the antidiuretic response which otherwise occurs in rats exposed to painful stimuli. The fact that such pretreatment does not influence significantly the antidiuretic response to injection of histamine or nicotine suggests that whereas the latter agents act directly in the hypothalamus, the effect of painful stimuli is mediated by more centrally placed mechanisms. This hypothesis is in accord with the data which indicate that chlorpromazine acts principally on the reticular system(8,9).

Summary. Administration of chlorpromazine inhibits the antidiuretic response of rats to painful stimuli but has no significant effect on antidiuretic response to injection of either histamine or nicotine.

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A Bivalent Live Virus Vaccine Against Canine Distemper (CD) and Infectious Canine Hepatitis (ICH).* (24242)

V. J. Cabasso, M. R. Stebbins and J. M. Avampato
(Introduced by Herald R. Cox)
Viral and Rickettsial Section, Research Division, Amer. Cyanamid Co., Pearl River, N. Y.

Cultivation in tissue culture of ICH virus (1) greatly facilitated its laboratory study, and opened the way for modification of its virulence to the dog. Such modification of the virus was reported, either by continuous serial passage in dog-kidney culture(2), or by propagation in swine-kidney cells(3,4,5). A combined CD-ICH vaccine containing swine-kidney-grown ICH virus recently was reported to be an effective immunizing agent against both diseases(6). This communication reports on attenuation of ICH virus after relatively few passages in swine kidney cultures, and on experiments on susceptible puppies with a bivalent CD-ICH vaccine.

Materials and methods. Virus strains. The CD strain used was a chick-embryo-adapted virus originally isolated in these laboratories from a field case of distemper encephalitis (7). Distemper challenge was carried out with a dog-brain suspension infected with Snyder Hill strain of canine distemper virus (8). The ICH virus used for both initiation of dog-kidney line of passages and challenge of vaccinated animals also was isolated in these laboratories, as reported earlier(9). *ICH virus. Cultivation, titration and serum neutralization.* Serial passages of the virus were made in Povitsky-bottle cultures of either dog- or embryonic-swine-kidney cells grown by the method of Dulbecco and Vogt (10). Growth fluid consisted of Earle's basal medium(11) containing lactalbumin hydrolysate and 15 or 20% normal calf serum. Just before inoculation, culture bottles were renewed with 100 ml of mixture 199(12). Virus titrations and serum neutralizations were carried out in stationary tubes of dog-kidney cells prepared as above. Renewal fluid consisted of 1 ml/tube of mixture 199 alone or of mixture 199 containing 2% horse serum. Titrations were made in 10-fold steps, by inoculating 0.1 ml of each dilution into 2 or 3 tubes. In serum neutralizations, mixtures of 2-fold dilutions of inactivated serum and 100-1000 TCD_{50} of virus also were inoculated in 0.1 ml volumes in 2 or 3 tubes, after incubation for 1 hour in 37°C waterbath. Titration and serum neutralization tubes were examined for 7 days, at the end of which 50% endpoints were calculated by the Reed and Muench formula (13). *CD virus. Cultivation and titration.* Both processes were carried out in 7- or 8-day chick embryos, using inoculation technic described by Gorham(14). For cultivation, a 10% suspension of infected chorio-allantoic membrane (CAM) was inoculated in 0.4 ml amounts/egg, with 0.2 ml dropped on the CAM at base of the natural air sac, and the remaining 0.2 ml introduced into the yolk sac. After 7 days of incubation at 36°C, both CAMs and embryos were harvested, pooled and made into a 30% tissue suspension. For virus titrations, 5-fold serial dilutions of vaccine were inoculated, each into 6 embryos, by dropping 0.2 ml volumes on the CAM. CAMs were examined 7 days later for characteristic lesions, and 50% endpoints were calculated. *Preparation of bivalent vaccine.* One part of undiluted, ICH-infected swine-kidney tissue-culture fluid was mixed with 9 parts of centrifuged (2000 rpm/20 minutes) 30% CD suspension. The mixture was distributed in glass vials in 2.4 ml amounts, and dried from frozen state. Vials were rubber-capped and aluminum-sealed under vacuum. Before its use in experiments, the vaccine was titrated for ICH and CD viruses, and tested on ferrets for potency of the CD component. *Complement fixation (CF).* The technics used, and the method of antigen preparation, were described earlier(15). *Experimental animals.* Litters

* The authors wish to express their appreciation to Mrs. K. Kiser for the CF tests and to Mrs. E. Chasan for help in preparation of the manuscript.

TABLE I. Puppy Virulence of ICH Virus Grown in Dog-Kidney Tissue Culture (Tests Performed 1953-1955).

Inoculation*		Clinical ICH			Results		
					Outcome		
Passage level	No. of puppies	No. typical	No. mild	Incubation	No. dead & time (days)	Inclusions in liver	C.F. antibodies in survivors
2–11	12	12		Usual	11 (3– 5)	11	1
13	1	1		"	1 (3)	1	
20	2	2		Prolonged	2 (14–27)	1	
30	2		2	Usual			2
50	3†						3
52	3†						3
	3‡		3	17–19 days			3

* All animals except those marked (†) and (‡) were inoculated with 0.1-0.15 ml undiluted tissue culture fluid in anterior chamber of right eye.
† Inoculated intraper. with 2 ml undiluted tissue culture fluid.
‡ Cage contacts to animals receiving 52nd passage virus.

of puppies of miscellaneous medium breeds, including mongrels, were secured from private sources. Puppies, with their mothers, were held in isolation from birth until they were weaned. They then were received in the laboratory and kept under careful observation for at least 2 weeks. Immediately preceding its use, each puppy was shown by the CF test to be negative for CD and ICH antibodies.

Results. I. Modification of ICH virus. A. Puppy virulence of ICH virus grown in dog kidney cultures. Over 18 months, each of various passage levels of the virus in dog kidney cultures was inoculated into 2 or 3 puppies. Virus was administered by one of 2 methods: either (a) 0.1-0.15 ml doses of infected undiluted tissue culture fluid were infiltrated into the anterior chamber of the eye of ICH susceptible puppies, a most sensitive route for ICH, or (b) 2 ml doses of undiluted fluid were inoculated intraperitoneally. Virus titers of different passage levels were of approximately the same order, varying between $10^{-5.0}$ and $10^{-6.0}$/0.1 ml. Temperature readings, leucocyte counts and blood sedimentation rate determinations were made daily on all inoculated puppies. Deaths from ICH were confirmed by presence in liver smears of intranuclear inclusions.

Results of these inoculations are summarized in Table I. To the 13th passage level puppies invariably developed within the expected incubation period, an acute form of the disease, with complete opacification of the inoculated eye. All animals but one (9th passage) died 3 to 5 days after inoculation. Beginning with 20th passage, incubation was more prolonged, death was unusually delayed, and intranuclear inclusions were found in only one puppy. By inoculation with the 30th passage only mild symptoms were produced within the usual incubation period, and both animals recovered and developed ICH antibodies.

A definite modification of the virus became noticeable with the 50th and 52nd passages, where no disease symptoms were recorded in any animal, although every one developed antibodies. However, puppies placed as cage contacts with those receiving the 52nd passage did come down with mild but definite ICH symptoms after 17-19 days of contact, but they recovered and acquired immunity to the disease.

B. Passage of ICH virus in swine-kidney tissue culture. The indication that a single puppy passage of the modified virus might cause substantial return of its virulence led to attempts to cultivate it in tissues of an unnatural host. Accordingly the 24th dog-kidney passage of the virus was successfully and serially propagated in embryonic swine-kidney tissue cultures. The 10th swine-kidney passage was subcutaneously inoculated into 3 susceptible litter-mate puppies, each receiving 1 ml of undiluted tissue culture fluid containing approximately 10^6 TCD_{50} of virus. Each puppy was housed in a separate cage, along with an uninoculated litter-mate to serve as

TABLE II. Response of Puppies to Falling Concentrations of Monovalent 14th Passage ICH Virus Grown in Embryonic Swine Kidney.

		Post-inoculation findings					Results of challenge†	
		Clinical						
			Corneal infiltration					
Puppy No.	No. of TCD_{50}*	Fever leukopenia	Dev't	Appearance (days)	Duration (days)	C.F. & SN antibodies at challenge	Clinical	Outcome
424	6.8	No	Yes	9	5	Yes	Normal	
425	Contact	"	No	—	—	No	Typical ICH	Died 6 days; inclusions +
426	5.8	"	"	—	—	Yes	Normal	
427	Contact	"	"	—	—	No	Typical ICH	Died 4 days; inclusions +
428	4.8	"	"	—	—	Yes	Normal	
429	3.8	"	"	—	—	"	"	
430	2.8	"	"	—	—	"	"	
420	1.8	"	"	—	—	"	"	
422	.8	"	"	—	—	"	"	
431 & 432		Challenge controls					Typical ICH	Recovered

* Expressed as log of power 10. Respective dilutions were inoculated in 2 ml volumes subcut.
† Virulent virus was administered subcut. 28 days after 1st inoculation.

contact control. A 5-week follow-up of the 6 animals revealed no disease symptoms whatever, except for a slight corneal cloudiness in both eyes of one inoculated puppy which appeared on the 9th day and cleared completely 5 days later. In this experiment challenge was not attempted, but the 3 inoculated puppies developed neutralizing antibodies in 1 to 2 weeks and CF antibodies in the 2nd to 3rd week. In contrast to experience with 52nd dog-kidney passage virus, cage contacts in this experiment remained normal throughout the 5 weeks of observation, and the only evidence of spread of swine-kidney virus was appearance of neutralizing antibodies in the 3 contacts 4 to 5 weeks after inoculation of their cage mates.

C. Antigenic extinction of swine-kidney-grown ICH virus. In an experiment to determine the puppy titer of swine-kidney virus, and to rule out any possibility of a "zoning effect" regarding its virulence, the 14th passage was used to inoculate a litter of 9 puppies. Each animal was inoculated subcutaneously with 2 ml of either the undiluted tissue culture fluid containing $10^{6.5}$ TCD_{50} of virus per ml or a serial 10-fold dilution thereof, up to 10^{-6}. The actual number of virus TCD_{50} injected ranged from 6 to 6,000,000. Animals were housed in separate cages, and with each of those inoculated with undiluted fluid or the 10^{-1} dilution, an uninoculated litter-mate was placed as contact control. After 28 days of observation the 9 inoculated puppies and 2 susceptible challenge controls were challenged subcutaneously with virulent ICH virus. All animals remained well until challenge, with the only deviation a slight opacification of one cornea in the puppy inoculated with 6,000,000 TCD_{50} (Table II). This corneal reaction started on the 9th day and by the 14th day had completely disappeared. Following challenge, all inoculated animals remained normal, while cage contacts and challenge controls developed typical ICH infection, the former dying in 4 to 6 days, the latter recovering.

D. Response of puppies in intra-ocular injection of swine-kidney-grown ICH virus. Further evidence of modification of the virus by its passage in swine-kidney cultures was obtained from the following experiment. Four susceptible puppies were inoculated by injection into the anterior eye chamber with 0.15 ml of 15th swine-kidney-tissue passage virus containing $10^{5.9}$ TCD_{50}. This route, known to be the most sensitive for ICH, terminates fatally in most cases of street ICH virus. Progress of infection in the 4 animals was fol-

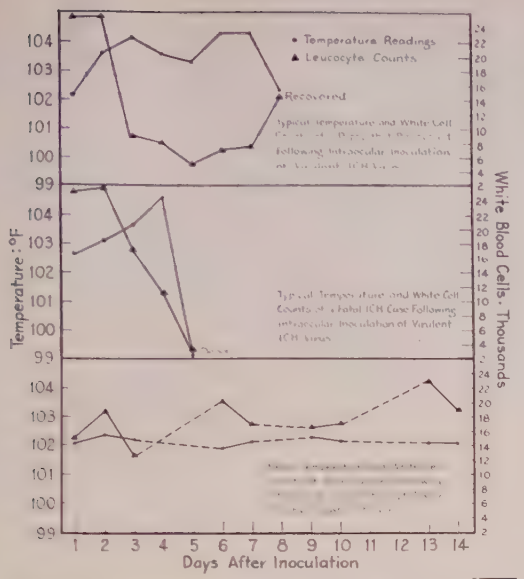

FIG. 1. Comparative response of puppies to intra-ocular inoculation of virulent and of swine-kidney-grown ICH virus as measured by temperature readings and white blood cell counts.

lowed by temperature readings and leucocyte counts. Beginning 3 to 4 days after inoculation, in all 4 puppies the injected eye became cloudy, and complete unilateral opacification subsequently developed and persisted throughout observation period. However, both temperatures and leucocyte counts remained within normal limits throughout the 20-day observation period, appetites of all animals remained normal, and they appeared to be in perfectly good health. For purposes of comparison, Fig. 1 shows mean temperature values and white cell counts of the 4 puppies for the first 2 weeks after inoculation contrasted with temperatures and cell counts in typical ICH cases, one fatal and one followed by recovery, elicited by intra-ocular administration of virulent ICH virus.

II. Evaluation of a combination of modified ICH and CD viruses. A combined vaccine was prepared, as described under Methods. After lyophilization it was found to contain 200 CED_{50} of CD and $10^{5.7}$ ICH doses/ml.

A. Response to CD component of bivalent vaccine of puppies immune to ICH. In one experiment each of 4 puppies that had survived the ICH test described in Table II was injected subcutaneously with 2 ml of the combined vaccine. Three other puppies that had survived the same test were left uninoculated to serve as cage contact controls. All animals remained normal in every respect for 48 days, when they were challenged intravenously with the Snyder Hill strain of distemper virus. The 4 vaccinated animals remained perfectly normal for one month, when they were put off test. In contrast, 2 of the 3 cage contacts developed typical disease symptoms, and died within 15 and 18 days of challenge, while the 3rd contact remained normal and survived.

B. Response of susceptible puppies to both components of bivalent vaccine, and urinary excretion of ICH virus. In another experiment, of a litter of 6 puppies susceptible to both CD and ICH, 3 were injected subcutaneously with 2 ml of bivalent vaccine and the other 3 were used as cage contact controls (Table III). In addition to usual daily determinations (temperature, leucocyte counts, blood sedimentation rate), every other day for 40 days urine specimens were collected from all 6 animals. For this a rather stressing method was employed: the animal was prevented from breathing by blocking its nostrils and mouth until it urinated (a period of several seconds up to 1 minute), when urine specimen was received in a sterile vessel. All animals remained normal during post-vaccination period, despite the stress of urine collection and daily heart punctures. Small quantities of virus were recovered from undiluted urine of vaccinated animals 18 or more days following injection. Virus recovery persisted in 2 animals for 1 to 3 days, and in the 3rd intermittently for 10 days. A questionable virus detection was made in 1 of the contact animals for a single day (25th after injection of its cage mate), while the other 2 remained negative. Forty-six days after vaccination, the 3 vaccinated animals, their 3 cage contacts, and 2 normal controls were challenged intraperitoneally with virulent ICH virus. The 3 vaccinated animals remained normal. One of the cage contacts developed a typical case of ICH and recovered, and the other 2 showed no disease symptoms. However, both challenge controls developed ICH and died.

TABLE III. Response of Puppies to Combined CD-ICH Vaccine. (Each 2 ml dose contained 400 CED_{50} of CD virus and $10^{6.0}$ TCD_{50} of ICH virus; ICH passage level was 15th in swine-kidney culture.)

Puppy No.	Status	Post-vaccination reactions	ICH virus in urine: Time of appearance (days)	ICH virus in urine: Duration (days)	Results of challenge*: ICH virus	Results of challenge*: CD virus
436	Vaccinated	None	18	2–3	Remained normal	Remained normal
440	Cage contact	"	Questionable 25	1	*Idem*	Sickened—died 26 days
437	Vaccinated	"	18	Intermittent 10	"	Remained normal
438	Cage contact	"	Negative		Typical ICH—recovered	Sickened—died 12 days
439	Vaccinated	"	27	1	Remained normal	Remained normal
441	Cage contact	"	Negative		*Idem*	Sickened—sacrificed 29 days
452	ICH challenge controls				Typical ICH—died 4 days	
453					Typical ICH—died 5 days	
468	CD challenge controls					Sickened—died 19 days
469						Sickened—recovered
470						Sickened—died 29 days
471						Remained normal

* ICH challenge took place 46 days after vaccination and was followed by CD 3 wk later.

Three weeks after ICH challenge the same 6 animals, with 4 normal controls, were challenged intravenously with virulent Snyder Hill distemper virus. The vaccinated animals again remained normal, but all 3 cage contacts developed the disease, 2 dying within 12 or 26 days and the 3rd being sacrificed in extremity on the 29th day. Of the 4 challenge controls, 3 sickened, 1 recovering and the other 2 dying within 19 or 29 days.

Discussion. Our experiments clearly show that virulence of ICH virus was profoundly modified in the course of its serial propagation in dog-kidney cultures and its subsequent continuous cultivation in embryonic swine tissue. When injected peripherally, the virus in its modified form seemed incapable of producing signs of disease in susceptible animals and caused no leucopenia. Although esthetically somewhat objectionable, the occasional cases of corneal opacification were mild and transient, and did not affect the general disposition of the animal. Even intra-ocular inoculation, despite prolonged involvement of the cornea, produced no systemic signs of disease. Following injection of the modified virus, immunity was elicited promptly and afforded solid protection against the virulent virus. It is interesting to note that 6 TCD_{50} of virus elicited as solid a resistance to challenge as 6,000,000 TCD_{50}.

The fact that, for a short time, the virus might be present in urine of vaccinated animals and could spread to intimate contacts does not seem to be of great concern, since no signs of increased virulence were noted even under conditions of severe stress. Spread of the immunizing agent could be detected only through serological tests or challenge, in marked contrast to the experience with the 52nd dog-kidney-passage virus (Table I).

The mixture of modified CD and ICH viruses seems to have no adverse effect on either agent, and in doubly susceptible animals it engenders good protection against both diseases. The bivalent vaccine was not prevented in the case of puppies already immune to ICH from producing an effective immunity to distemper.

Summary. The virulence of ICH virus has

been effectively modified by serial propagation in dog-kidney cultures followed by adaptation to and serial cultivation in swine-embryo tissue. The modified virus is an effective immunizing agent, and produces in the vaccinated animal no adverse reaction except for an occasional case of light and transient opacification of the eye following massive peripheral injection of the virus. The modified ICH virus may spread through the urine of vaccinated animals to intimate contact animals, but although these develop antibodies they show no signs of disease. A combined vaccine prepared by mixing modified CD and ICH viruses elicited good protection against both diseases in doubly susceptible animals, and in puppies already immune to ICH did not interfere with the production of immunity to CD.

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Synthesis of Cholesterol by a Strain of Human Uterine Fibroblasts Propagated *in vitro*. (24243)

DAVID L. BERLINER,* H. EARLE SWIM,† AND THOMAS F. DOUGHERTY
University of Utah Medical School and Western Reserve University School of Medicine

It is generally accepted that fibroblasts constitute a high proportion of cells in loose connective tissue(1). The metabolic capabilities of fibroblasts appear to be related in some fashion to their high degree of resistance to the action of antiphlogistic steroids(2). These observations might be related to the fact that fibroblasts appear to play an important role in extrahepatic metabolism of steroids(3,4). It has also been demonstrated that strains of U12 fibroblasts isolated from human myometrium and propagated *in vitro* for many years have the capacity to metabolize cortisol and progesterone to a variety of steroid products(5). These observations raised the question as to whether U12 cells might also be able to synthesize cholesterol and possibly other steroids from low molecular weight precursors such as acetate. The synthesis of cholesterol could not be evaluated on the basis of nutrition since the least fastidious strain of U12 (6) will not proliferate in media devoid of dialyzed horse serum. The data to be presented indicate that strain U12-79 is capable of synthesizing cholesterol as judged by appearance of isotope in this compound when the cells are grown in a medium containing C^{14} acetate.

Experimental. Stock cultures of U12-79 were propagated in a chemically defined solu-

* Key Investigator, Am. Cancer Soc.

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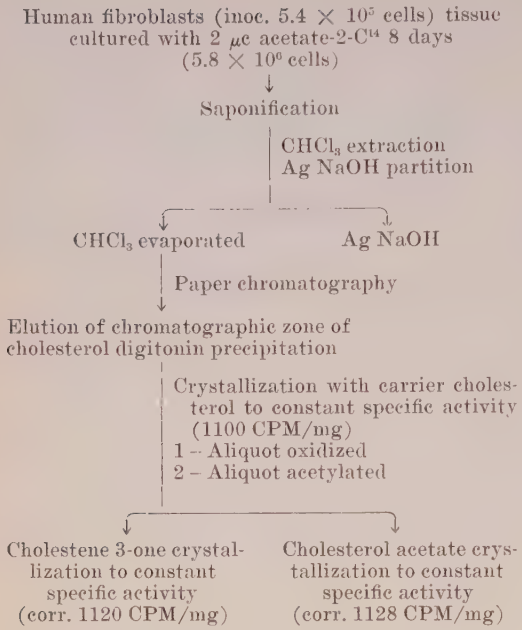

FIG. 1. Procedure for the identification of C^{14} cholesterol U12-79.

tion, S103(7), supplemented with 5% by volume of dialyzed horse serum according to methods described by Swim and Parker(8). To ascertain whether cholesterol was synthesized by U12-79, the cells were propagated in this medium supplemented with 0.17 μM/ml of acetate-2-C^{14} (specific activity, 1 μc/μM) as follows. Ten T60 flasks were planted with 5.4 x 10^5 cells/flask. On the 4th day the fluid was removed from each flask and replaced with fresh medium. On 8th day the medium was again removed and cells were washed twice with Earle's saline(9). At this time, the average number of cells/flask was 5.8 x 10^6. The medium from 4th and 8th day and the Earle's saline were combined. The pooled medium and the cells were analyzed separately for cholesterol as outlined in Fig. 1. The cells were saponified and the resulting mixture extracted 3 times with chloroform. The combined chloroform extract was evaporated and the residue was chromatographed directly on paper, using a modification of the hexane system of Zaffaroni (10), in which the formamide is not applied to the paper. That is, the sample is placed directly on the paper in hexane, and the chromatogram is developed with this solvent as the mobile phase. Under these conditions, cholesterol has an R_f of 0.65. The chromatographic zone corresponding to cholesterol was eluted with MeOH=$CHCl_3$(1:1). Non-radioactive carrier cholesterol was added to the eluate and cholesterol was precipitated with digitonin. The digitonin complex was hydrolyzed and the resulting cholesterol was crystallized to constant specific activity of 427 cpm/μM. A portion of this cholesterol was converted to cholestene-3-one by treatment with chromic acid. The cholestene-3-one after crystallizing to constant specific activity contained 435 cpm/μM. A second sample of purified cholesterol was acetylated and the resulting cholesterol acetate upon crystallizing to constant specific activity contained 428 cpm/μM. C^{14}-cholesterol was also isolated from the medium in this experiment and identified by the same procedures. The total C^{14} found in the cholesterol isolated from the cells and medium was approximately 0.8% of that added to the medium initially as acetate-2-C^{14}.

Discussion. The foregoing data indicate that the sequence of reactions leading to synthesis of cholesterol from acetate is operative in strain U12-79. Although the present experiments do not provide information on the extent to which net synthesis occurs, they indicate that these cells are capable of synthesizing cholesterol. Labeled cholesterol is found in a variety of tissues after intact animals are administered isotopic acetate(11); however, it is difficult to distinguish the cellular site of origin in tissues due to the mixed cell population. In this connection, it is of interest that cells of mesenchymal origin are capable of synthesizing cholesterol after 5 years *in vitro*. In view of the difficulties and uncertainties involved in studying the factors concerned with accumulation of cholesterol in various mammalian cells in the intact animal, the U12-79 system appears to offer a number of advantages as a model system for investigations of this type. In the course of these experiments other labeled compounds were isolated, some of which appear to be steroidal in nature. Experiments are under way at the present time to identify the sterol type com-

pounds in an attempt to relate some of the anabolic and catabolic aspects of steroid metabolism by strain U12-79.

Summary. Strain U12-79 of human uterine fibroblasts propagated *in vitro* for almost 5 years was grown in a medium containing acetate-2-C^{14}. C^{14}-cholesterol was isolated from both the cells and the medium.

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Rate of Flow and Cell Count of Thoracic Duct Lymph in the Mouse. (24244)

MARVIN M. SHREWSBURY (Introduced by William O. Reinhardt)
Dept. of Biology, San Jose State College, San Jose, Calif.

This report describes a method for collection of thoracic duct lymph in anesthetized mice, and presents data on rate of flow, white cell count, and total lymphocyte output of lymph obtained. These data are compared with similar measurements of thoracic duct lymph in other animal forms.

Technic. Male mice (40 days old) of NIH-Webster strain, unfasted, 17-23 g B.W. were anesthetized by intraperitoneal injection of 6% sodium pentobarbital (7 mg/100 g B.W.). An incision was made through the abdominal wall, following the usual dorsal approach for left adrenalectomy. Manipulations and lymph collection were performed in a bloodless field under a dissecting microscope. The incised body wall was retracted to expose the abdominal aorta. Viscera within the field were carefully retracted, care being taken to avoid entering the peritoneal cavity. The aorta, cranial to the mesenteric lymph node (Aselli), was displaced gently away from posterior abdominal muscles caudal to the diaphragm. This procedure exposed the thoracic duct, contiguous to posterior aspect of the aorta. The dissection, carefully executed, resulted in formation of a depression or cup, bounded by the aorta on the left side and paravertebral muscles on the right. The superior border of this cup was demarcated by the aorta as it traversed the diaphragm; the inferior border of the cup was formed by juncture of the aorta, mesenteric lymph node, and paravertebral muscles. The floor of the cup was formed primarily by the left side of the thoracic duct.

The point of a 27 G hypodermic needle was used to make 2 slits in the exposed thoracic duct. The first opening was cephalic to a segmental artery crossing the duct. To insure complete drainage of the thoracic duct lymph into the structural cup, a second opening was made caudal to this segmental artery. After incising the wall of the duct, a free flow of clear or milky lymph drained into the anatomical cup or depression. The tip of a fine glass sequestrene treated cannula of small bore was then placed in the cup adjacent to the duct incisions. Lymph was drained through the cannula into a small glass vial under slight negative pressure. Lymph was collected for half hour periods, measurements being made at intervals during collection.

Results. Table I presents pertinent data

TABLE I. Thoracic Duct Lymph Flow and Lymphocyte Output in Anaesthetized Male Mice.

Exp.	No. of animals	Body wt, g	Collection time, min.	Lymph vol ml/hr	Lymph vol ml/hr/kg B.W.	Lymphocyte Content, cells/mm³ × 10³	Lymphocyte Output Cells/hr × 10⁶	Lymphocyte Output Cells/hr/kg B.W. × 10⁶
Controls	20	20 ± .5*	90	.10 ± .01	5.5 ± .7	11.7 ± 1.1	1.1 ± .2	57.0 ± 8.4
Diurnal variation								
A.M.	6	20 ± .6	90	.10 ± .02	4.9 ± 1.0	6.8 ± 1.2	.7 ± .1	34.6 ± 6.5
P.M.	5	19 ± .4	90	.11 ± .03	6.1 ± 1.3	18.6 ± 2.7	1.9 ± .3	104.6 ±15.6

* ± S.E.

determined on thoracic duct lymph from 20 anesthetized mice.

Lymph flow from the thoracic duct lymph varied in quantity from 0.03-0.24 ml/hr. Lymph flow remained fairly constant for each animal during collection, although the cell output had a tendency to rise during later sampling periods. The mean flow (ml/hr/kg B.W.) was greater than that reported for most other animals; the highest values obtained for lymph flow in the mouse (14 ml/hr/kg B.W.) were greater than recorded for other animals under similar conditions(1). There appeared to be no consistent relationship between lymph flow, lymphocyte output, and body weight. On the basis of average thoracic duct lymph flow of 0.1 ml/hr, it can be calculated that a volume of at least 2.4 ml of thoracic duct lymph passes into the venous blood in 24 hr period. This amounts to approximately one and one-half times the total blood volume(2). White cell counts for the thoracic duct lymph averaged 11,700 ± 1,100 and varied from 1,000-35,400 cells/mm³. The mean value for the thoracic duct lymphocyte output (cells/hr/kg B.W.) was similar to values obtained for the guinea pig(1).

Comparison of the values for lymph collections obtained from 9:30-10:30 a.m. with similar data obtained from 3:30-4:30 p.m. was used to determine extent of diurnal variation. A diurnal variation was apparent for average total lymphocyte output (cells/mm³, cells/hr, and cells/hr/kg B.W.) from the thoracic duct for mice in which lymph was collected for the morning hours, as compared to a similar collection period in the afternoon. The data showed a significantly higher value ($p < .01$) for mean cell count and output in the afternoon period.

Protein concentration as measured by the copper sulfate specific gravity method averaged 4.6 ± 0.3 g% for the thoracic duct lymph of 9 mice.

Summary. 1. A method is described for obtaining lymph from the abdominal thoracic duct of the anesthetized mouse. 2. Average rate of lymph flow was approximately 0.1 ml/hr, or 5.5 ml/hr/kg B.W. 3. The cell content averaged 11,700/mm³, and the total lymphocyte output averaged 1.10 x 10⁶/hr. 4. There was a significant diurnal variation in lymphocyte output which was greater in the afternoon than in the morning.

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Effects of Cortisol and Phenylbutazone on Experimental Arthritis and Serum Polysaccharide/Protein Ratios in Rats. (24245)

Lee G. Hershberger (Introduced by F. J. Saunders)
(With technical assistance of Nancy Hedl, Laura Hansen and Elaine Gochman)
Division of Biological Research, G. D. Searle & Co., Chicago, Ill.

Jasmin observed that exudate of the Murphy rat lymphosarcoma (MRLS) tumor-pouch will produce typical proliferative arthritis in rats(1). The articular lesions, which show heavy cellular infiltrations in the synovia, pannus-like proliferations and erosion of the articular cartilage(2), develop in 3-7 days and are aggravated by adrenalectomy(3). Since the polysaccharide/protein (PR) ratio of serum correlates well with clinical activity of rheumatoid arthritis(4), it was of interest to study PR ratios in rats with this type of experimental polyarthritis.

Materials and methods. Male Sprague-Dawley rats were maintained on Rockland rat diet and tap water *ad lib.* The MRLS exudate was prepared according to the method of Jasmin(2). Briefly, a tumor cell suspension was prepared and 0.5 ml was injected into a 25 ml pneumodermal pouch on dorsal side of male rats. After 7-10 days the exudate was withdrawn from the pouch, centrifuged, and either used immediately or after 1 week's storage at −5°C. One ml of this exudate was administered intraperitoneally to male rats weighing about 180 g, 24 hours after adrenalectomy. Each rat received a 0.1 mg maintenance dose of desoxycorticosterone acetate (DCA) daily. Daily therapy with cortisol or phenylbutazone, given subcutaneously in oil, was begun at time of exudate administration and continued for 5 treatments. One day after the last treatment, blood samples were taken by cardiac puncture and the animals sacrificed for macroscopic examination of the joints. Three regions were examined for swelling on each extrmity: paw, tibiotarsal or radio-carpal joint, and femuro-tibial or humero-radial joint. Severity of lesions was graded on a scale of 0 to ++++. Serum nonglucosamine polysaccharide concentrations were determined by the method of Shetlar *et al.*(5) and total serum protein was determined by the biuret method. The PR ratio of Payne *et al.*(6) was obtained from the following relationship:

$$PR = \frac{[\text{serum polysaccharide}]}{[\text{serum protein}]} \times 100.$$

Results. None of the control rats developed signs of polyarthritis, whereas 95% of the animals that received the MRLS exudate, but no therapy, and survived the treatment period, showed swelling of one or more of the sites (Table I). Only 22% of the rats that received cortisol at 1 mg were affected ($P < 0.05$). None of the animals in the 5 mg cortisol group showed any swelling ($P < 0.01$). Phenylbutazone, on the other hand, had no significant effect on incidence of arthritis ($P > 0.05$). Severity of lesions in afflicted animals was reduced significantly by cortisol treatment, but was not decreased significantly with phenylbutazone. The mortality of 28% produced by MRLS exudate was eliminated by treatment at both dose levels of cortisol, but was not reduced significantly with phenylbutazone.

MRLS exudate clearly increased serum polysaccharide levels without producing any great change in protein concentrations (Table I). PR ratios, therefore, were increased in all groups receiving exudate (Fig. 1). Cortisol treatment increased protein levels of both treated and control animals. Polysaccharide values were not reduced significantly by cortisol. Cortisol decreased the PR ratio in the rats treated with exudate and the 5 mg dose level appeared to reduce the PR ratio in the control animals. Phenylbutazone treatment produced no consistent changes in serum polysaccharide, protein, or PR ratio.

Discussion. These experiments confirm the work of Jasmin on production of polyarthritis in rats with a single injection of MRLS exudate and the inhibition of arthritic lesions with cortisol(2). In addition, it was

TABLE I. Effects of Cortisol and Phenylbutazone on Polyarthritis and Serum Changes in Rats Treated with MRLS Exudate.

Treatment	Incidence of swelling in survivors		Severity of swelling in afflicted rats	Mortality	Blood serum				
	%	Rats			Rats	Polysaccharide, mg %		Protein, %	
(A) Control									
Oil	0	0/13	0	1/14	13	150	4*	5.64	.08*
Cortisol, 1 mg	0	0/ 5	0	0/ 5	5	169	5	6.13	.06
" , 5 "	0	0/ 5	0	0/ 5	4	152	3	6.46	.06
Phenylbutazone, 20 mg	0	0/ 7	0	3/10	7	168	6	5.66	.08
(B) MRLS exudate									
Oil	95	19/20	2.5	8/28	13	228	6	5.91	.15
Cortisol, 1 mg	22	2/ 9	1.0	0/ 9	5	238	11	6.68	.11
" , 5 "	0	0/20	0	0/20	13	210	9	6.91	.15
Phenylbutazone, 20 mg	71	10/14	2.1	4/18	9	217	10	5.56	.17

* Stand. error of mean.

shown that phenylbutazone did not significantly inhibit incidence or severity of the lesions in our experiments. Phenylbutazone is weakly active, at best, in most other animal tests for anti-inflammatory activity and is metabolized rapidly in the rat(7).

Administration of MRLS exudate resulted in polyarthritis and an increase in PR ratio which was attributable to a relative increase in polysaccharide. These facts would suggest that the increased polysaccharide was associated somehow with the arthritis. Cortisol treatment reversed the shift in PR ratio mainly by increasing protein levels in the serum, suggesting that the increase in protein might have been associated with inhibition of MRLS arthritis. However, cortisol at the 5 mg level appeared to decrease PR ratio in the control rats, mainly by increasing serum protein. This might be considered the result of the "protein mobilizing" effect of cortisol(8), rather than the anti-inflammatory effect. It is of interest that both MRLS arthritis in the rat and rheumatoid arthritis in man are associated with increases in serum PR ratio and that cortisol reduces the PR ratio in both conditions. Further work will be required, however, to determine reasons for the association between inflammatory diseases and shifts in PR ratio of the serum. Winzler has discussed 5 hypotheses explaining the rise in serum glycoproteins during inflammation(9).

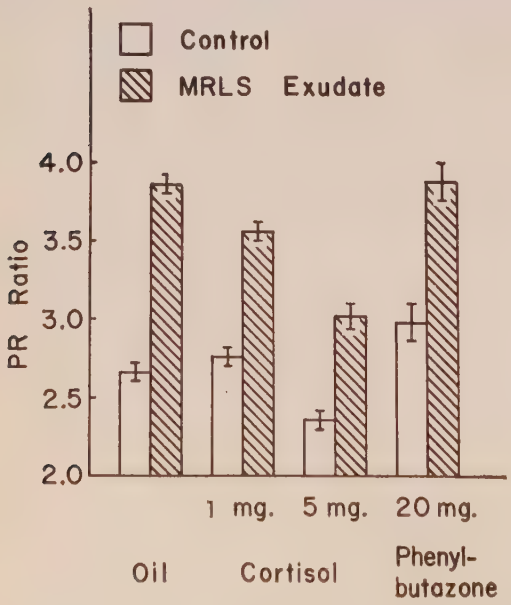

FIG. 1. Effects of cortisol and phenylbutazone on serum PR ratios in rats treated with MRLS exudate. Bars represent stand. error.

It is possible that tumor cells could have been transferred into the experimental animals in the tumor exudate even though the exudate had been centrifuged and no tumors were found at autopsy after 5 days. If so, this might explain the changes in PR ratio since neoplastic diseases are known to affect PR ratio(6). The observation that endotoxins increase glycoprotein levels in the serum(10) suggests another possible explanation for the shifts in PR ratio observed in our experiments. Jasmin has presented evidence that the arthritogenic agent in MRLS exudate is a microorganism growing in the tumor(2). Thus,

the shifts in PR ratio could have been the result of an infection rather than of polyarthritis, *per se*.

The animals treated with exudate plus cortisol were in much better physical condition than the animals treated with exudate alone as evidenced by appearance of the coat, activity of the animals, and mortality figures (Table I). This would indicate that under the conditions of these experiments the adrenal replacement (or anti-stress) effects of cortisol and the anti-inflammatory effects of cortisol were dominant over the pro-infective effects.

In subsequent experiments, using new batches of MRLS exudate, we were unable to reproduce the expected incidence of polyarthritis. If the arthritogenic factor in the exudate used earlier was an organism growing in the tumor as Jasmin's work suggests, perhaps we lost our "culture" during serial transplantations of the tumor.

Microscopic examination of the joints after 5 days suggested that the inflammatory reactions originated in the periarticular tissues. Conclusive proof of the site of origin will require sectioning the joints at earlier stages of the reaction and perhaps examination of serial sections. Regardless of whether the lesions originate in the periarticular or the synovial tissues, the severe inflammatory reaction produced by MRLS exudate offers a promising approach to study of inflammation and anti-inflammatory drugs.

Summary. A single injection of exudate from Murphy rat lymphosarcoma produced polyarthritis and elevated polysaccharide/protein ratios of the serum. In a later series of experiments the incidence of arthritis was much less. When arthritis was produced cortisol inhibited the incidence and severity of the arthritic lesions and partially reversed the shift in polysaccharide/protein ratio. Phenylbutazone had no consistent effect on polyarthritis or serum ratios.

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Response to Exogenous Gonadotrophins in Absence of Dietary Protein.* (24246)

HERBERT H. SREBNIK, MARJORIE M. NELSON AND MIRIAM E. SIMPSON
Institute of Experimental Biology and Department of Anatomy, University of California, Berkeley

Rats on low-protein or protein-free diets exhibit marked disturbances of the reproductive system as shown by prolonged anestrus, ovarian and accessory organ atrophy and by failure to maintain pregnancy(1,2). Estrone and progesterone at dosage levels effective in rats hypophysectomized and ovariectomized soon after breeding(3) successfully maintained pregnancy in the absence of dietary

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protein(4). The gonadal atrophy resulting from such diets suggested a reduction in circulating gonadotrophic hormones or an impairment of ovarian sensitivity. This study was designed to test the response of female rats to administration of various gonadotrophins in the complete absence of dietary protein.

Methods. Female rats (Long-Evans strain) approximately 80 days of age and 200 g in body weight were fed a purified diet, free of protein, but containing all other known dietary essentials.† Vaginal smears were examined daily, and the animals weighed every 5 days. After 5 weeks on the protein-free diet, the rats were distributed into equivalent groups of 3 to 5 rats according to body weight and length of anestrus. When injection of gonadotrophins was begun the rats had been in anestrus for approximately a month and had lost one-third of their initial body weight. Four gonadotrophic hormones‡ were injected: pituitary follicle stimulating hormone (FSH) and interstitial cell stimulating hormone (ICSH), human chorionic gonadotrophin (HCG) and equine gonadotrophin (PMS). Varying levels of FSH, ICSH and HCG were given subcutaneously once daily for 4 successive days; PMS was administered in a single subcutaneous dose. As a standard of reference for response to hypophyseal gonadotrophins, hypophysectomized rats, 17-20 days post-operative, were used. These rats were similar in age and body weight to the protein-deficient animals and were maintained on a stock diet of natural foodstuffs.§ In experiments involving non-pituitary gonadotrophins normal immature rats,‖ 24 days of age, were injected parallel with protein-deficient animals. Hypophysectomized rats are less responsive to low levels of HCG and PMS(6) than intact animals and, therefore, are not suitable as controls for these gonadotrophins. All rats were autopsied 96 hours after onset of injection. Uteri were freed of their oviducts, drained of fluid and weighed. Ovaries were weighed, fixed in Bouins solution and stained with hematoxylin and eosin for histological study. Criteria for ovarian stimulation were those described by Evans *et al.*(7). Follicle stimulating activity was determined by resumption of follicular growth, and interstitial cell stimulating activity by repair of the atrophic interstitial cells, *i.e.*, return to normal nuclear pattern and cell size. Production of fully mature follicles and of corpora lutea as well as uterine response gave additional evidence of ovarian sensitivity. The critical dosage levels for minimal response to each gonadotrophin were confirmed at least once.

Results. Follicular development was about equally retarded in the ovaries of uninjected protein-deficient and of adult hypophysectomized rats, both containing only small follicles. Follicular development was perhaps slightly greater in protein-deficient rats, beginning antrum formation being observed occasionally. Persistent corpora lutea were present in the ovaries of both types of rats. The interstitial cells in the protein-deficient animals had the same morphological and staining characteristics as in hypophysectomized rats ("wheel cells"), though they appeared to be increased in number.

Pituitary *FSH* had no effect on follicular growth in protein-deficient or in adult hypo-

† The protein-free diet consisted of sucrose 88%, hydrogenated cottonseed oil 8%, salts No. 4(5) 4%. Crystalline vitamins/kg of diet were: vit. B_{12} 0.05 mg, *d*-biotin 0.6 mg, 2-methyl 1,4-naphthoquinone 10 mg, thiamine HCl 10 mg, pteroylglutamic acid 11 mg, riboflavin 20 mg, p-aminobenzoic acid 20 mg, niacin 40 mg, d-calcium pantothenate 100 mg, inositol 800 mg, choline chloride 1000 mg. All rats received weekly a fat-soluble vitamin supplement of 800 USP units vit. A, 115 chick units vit. D, 6 mg synthetic alphatocopherol and 650 mg corn oil (Mazola).

‡ FSH prepared from sheep pituitaries and ICSH prepared from beef pituitaries by ammonium sulfate fractionation; HCG ("Antophysin"), prepared by Winthrop Laboratories; PMS ("Gonadin"), prepared by Cutter Laboratories.

§ Diet I is composed of wheat 67.5%, casein 15%, skim milk powder 7.5%, hydrogenated vegetable oil 6.75%, fish oil 1%, iodized NaCl 0.75%, $CaCO_3$ 1.5%. This was supplemented every day by a wet mash of the same diet.

‖ Normal immature rats received the regular stock diet (Diet XIV) consisting of wheat 68.5%, casein 5%, fish meal 10%, alfalfa leaf meal 10%, fish oil 5%, iodized NaCl 1.5%. Lettuce was given 2-3 times weekly.

TABLE I. Response to Hypophyseal FSH in Protein-Deficient and in Hypophysectomized Adult Rats.

Total dose, RU*	No. of rats	Ovaries				Uterine wt, mg	
		Wt, mg		Follicles†	Interstitial cells		
		Protein-deficient					
0	11	25	2‡	s	Atrophic	80	4‡
1	3	26	5	s	"	77	8
2	8	30	4	sm	"	84	4
4	11	34	2	m	"	94	8
8	8	44	3	m–l	"	167	18
		Hypophysectomized					
0	11	29	2	s	Atrophic	68	3
1	9	29	3	s	"	68	4
2	9	35	3	sm	"	81	10
4	9	44	2	m	"	95	13
8	9	54	5	m–l	"	170	12

* 1 RU (rat unit), as defined by Evans *et al.*(7), represents minimal total amount which, inj. subcut. over a 3-day period, caused resumption of follicular growth in rats hypophysectomized at 26-28 days of age and 6-8 days post-operative.

† Follicles were measured with ocular micrometer and designated as small (s) = 375 μ, small medium (sm) = 450-500 μ, medium (m) = 550-650 μ, medium-large (ml) = 700-750 μ, and large (l) = 800-1000 μ.

‡ ± stand. error of mean.

physectomized rats when 1 rat unit (RU) was given (Table I). Both groups responded, however, to 2 RU with a slight increase in follicle size and beginning antrum formation. With the highest dosage, 8 RU, uterine weights were increased to the same extent. No effect on the atrophic interstitial tissue was observed in either type of rat up to the 8 RU dose level. Injection of ½ RU of pituitary *ICSH* in protein-deficient rats (Table II) caused a detectable increase in the amount of interstitial cell cytoplasm and in the intensity of the staining reaction (designated as "partial repair"). Further increase in cell size and staining intensity, as well as increase in nuclear size and some scattering of the clumped chromatin, were observed with 1 RU ICSH. Hypophysectomized rats required twice as much ICSH, namely 1 RU and 2 RU respectively, for similar responses.

HCG resulted in follicular growth and increased uterine weights in protein-deficient rats at 1.25 IU (Table III); 2.5 IU HCG induced corpus luteum formation and partial repair of interstitial tissue. In normal immature rats, used in comparisons of response to

TABLE II. Response to Hypophyseal ICSH in Protein-Deficient and in Hypophysectomized Adult Rats.

Total dose, RU*	No. of rats	Ovaries				Uterine wt, mg	
		Wt, mg		Follicles	Interstitial cells		
		Protein-deficient					
0	24	25	1†	s	Atrophic	79	3†
.25	16	30	2	"	"	95	5
.5	16	29	1	"	Partial repair	86	3
1.0	16	28	1	"	*Idem*	88	2
		Hypophysectomized					
0	16	31	2	s	Atrophic	72	4
.5	7	29	1	"	"	84	5
1.0	15	29	2	"	Partial repair	88	5
2.0	16	32	2	"	*Idem*	87	3

* 1 RU (rat unit), as defined by Evans *et al.*(7), represents minimal total amount which, inj. intraper. over a 3-day period, caused repair of "deficient" interstitial cells in rats hypophysectomized at 26-28 days of age and 6-8 days post-operative. When ICSH is inj. subcut., as in this study, approximately 4 times as much of the hormone is required.

† ± stand. error of mean.

TABLE III. Response to HCG in Protein-Deficient and in Normal Immature Rats.

Total dose, IU	No. of rats	Ovaries: Wt, mg		Ovaries: Follicles	Ovaries: Interstitial cells	Uterine wt, mg	
		Protein-deficient					
0	11	26	2†	s	Atrophic	83	4†
.3–.6	6	26	1	s	"	108	18
1.25	8	29	2	sm, m	"	185	20
2.5	8	38	3	m–ml, CL*	Partial repair	185	10
3.75–5.0	6	41	3	m–l, CL	Repair	164	9
		Normal immature					
0	10	19	1	sm, few m	Epithelioid	22	1
.6	10	19	1	*Idem*	"	28	2
1.2	10	18	2	"	"	23	2
2.5	10	18	1	ml, few l	"	116	18
5.0	5	28	3	few ml, CL	"	102	6

* Newly formed corpora lutea. † ± stand. error of mean.

this gonadotrophin, 1.25 IU caused no follicular development, 2.5 IU being required for follicular and uterine growth and 5 IU for corpus luteum formation. Only 0.25 IU of *PMS* was needed to stimulate follicular development and increase uterine weights in protein-deficient rats (Table IV), and 0.5 IU resulted in corpus luteum formation and partial repair of the interstitial tissue. In normal immature rats higher levels of PMS, namely 0.5 - 1 IU, were necessary for follicular enlargement and uterine response, and 1 IU for corpus luteum formation.

Discussion. It has been shown in this study that the ovaries of protein-deficient rats responded to exogenous gonadotrophins. Pituitary FSH and ICSH were at least as effective as in hypophysectomized rats of similar age and body weight, whereas HCG and PMS were twice as effective as in normal immature rats. Starvation or food restriction, conditions in which protein consumption is correspondingly reduced, did not interfere with the response of the ovaries to pituitary or nonpituitary gonadotrophins (*e.g.* 8,9,10,11). Similar results were obtained in lysine deficiency(12). The effects of small amounts of hormone, or of graded doses, however, have not been adequately explored; moreover, no purified hypophyseal gonadotrophins have been tested during inanition and their effects compared with those in hypophysectomized animals.

Ovarian responsiveness to gonadotrophins, under conditions comparable to those used here, has been investigated during Vit. B_6 deficiency(13). B_6-deficient rats required 8 times as much FSH and twice as much ICSH for follicular stimulation and interstitial repair as hypophysectomized rats of comparable age and body weight. The FSH and ICSH preparations were identical with those

TABLE IV. Response to PMS in Protein-Deficient and in Normal Immature Rats.

Total dose, IU	No. of rats	Ovaries: Wt, mg		Ovaries: Follicles	Ovaries: Interstitial cells	Uterine wt, mg	
		Protein-deficient					
0	14	25	2*	s	Atrophic	89	2*
.1	9	28	2	s	"	101	7
.25	9	29	2	sm	"	188	19
.5	14	37	2	m–l, CL	Partial repair	204	12
1.0	10	42	3	m–l, CL	Repair	160	12
		Normal immature					
0	19	17	1	sm, few m	Epithelioid	27	2
.5	14	16	1	sm, few m–ml	"	63	12
1.0	15	25	1	few m, ml, CL	"	110	3

* ± stand. error of mean.

used in this study. It would appear, therefore, that loss of ovarian function in B_6 deficiency may be partly due to impaired ovarian sensitivity. In protein-deficient rats, where no such impairment exists, reduction of circulating gonadotrophins is a more likely explanation. Lack of circulating gonadotrophins has previously been suggested as an explanation for the gonadal atrophy resulting from inanition(8,9,10).

The reason for the greater effectiveness of HCG and PMS in protein-deficient rats than in normal immature rats is not clear. These hormones may be more rapidly inactivated or excreted in normal immature rats, or the ovaries of sexually immature animals may be refractory to low levels of these gonadotrophins. Both HCG and PMS exert gonadotrophic action at lower doses in normal than in hypophysectomized rats(6). It is commonly assumed that these hormones stimulate the pituitary in the normal rat, the FSH released acting synergically with the ICSH-like component of the injected hormone. Follicular growth following injection of HCG and PMS into protein-deficient rats might then result from a similar mechanism. The pituitaries of protein-deficient rats have already been shown by bioassay to contain FSH (14), and this may be released under the stimulus of these hormones.

Summary. Adult female rats were given a protein-free diet for 5 weeks and their response to graded amounts of gonadotrophic hormones determined. Protein-deficient rats responded to FSH with follicular growth at the same dosage levels as adult hypophysectomized rats of similar age and body weight. ICSH, as judged by stimulation of interstitial tissue, was slightly more effective in protein-deficient than in hypophysectomized rats. HCG and PMS induced follicular growth, corpus luteum formation and increased uterine weights in protein-deficient rats at one-half the dosages necessary in normal immature rats.

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Effect of Pre-Feeding of Fat on I^{131} Triolein Absorption in Subtotal Gastrectomy Patients. (24247)

MALCOLM P. TYOR AND JULIAN M. RUFFIN
(Introduced by E. A. Stead)
Departments of Medicine, Duke University Medical Center, Veterans Administration Hospital, Durham

Oral administration of I^{131} labeled triolein to patients with subtotal gastrectomy (Billroth II) frequently yields abnormal results consisting of reduced levels of blood radioac-

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tivity and increased concentrations of radioactive material in the stool(1). Blood and fecal values of radioactivity obtained from the same patients given I^{131} labeled oleic acid have been normal in most instances, indicating that the defect exhibited is primarily one of digestion of triglyceride rather than absorption of fatty acid(1). Following *intraduodenal injection* of I^{131} labeled lipids (triolein and oleic acid), similar results for blood and fecal radioactivity have been found in normal subjects(2). Recently, this induced defect in normal subjects has been partially or completely corrected by oral or intraduodenal administration of untagged lipid emulsion given prior to intraduodenal infusion of tagged triolein (unpublished). Since the results following oral I^{131} lipid administration to patients with subtotal gastrectomy were similar to those observed in normal subjects following duodenal intubation, an untagged lipid meal was given to patients with subtotal gastrectomy prior to administration of tagged triolein.

Methods. Twelve male patients, age 30 to 72 years, who had had a subtotal gastrectomy (Billroth II) 2 to 9 years previously were studied. In no instance was there clinical or laboratory evidence to suggest an associated disorder of pancreas or small intestine. Each patient was studied twice. The first test was conducted in the fasting state; 58 to 80 ml of a lipid emulsion, which contained I^{131} labeled triolein being administered orally. The second study period began 96 to 168 hours after the first and was similar in every respect, except that 50 ml of non-radioactive lipid emulsion (peanut oil and water) was given orally 30 minutes prior to second test dose of tagged lipid. The methods of preparation and administration of the I^{131} labeled triolein emulsion as well as collection and counting of blood and fecal samples have been previously described(3). Blood and fecal radioactivity were expressed as percentage of administered dose/liter of whole blood and 48 to 72 hour collection period, respectively (3). Blood samples were obtained at hourly intervals for 6 hours. Fecal samples were collected for 24 to 72 hours and were weighed. Liquid stools were judged to be possibly contaminated with urine and were discarded. Blood radioactivity data were analyzed by comparing differences between means of paired values obtained at each sampling time during the 2 study periods.

Results. (Table I). When patients were pre-fed with lipid, concentrations of blood radioactivity measured 3 to 6 hours after labeled lipid drink were significantly greater, $P = <0.01$, than previous values obtained after labeled triolein was given without lipid pre-feeding. Blood values obtained at 1 and 2 hours were similar for both study periods, as was the time of maximum blood radioactivity, $P = >0.20$.

Fecal radioactivity was increased in 10 patients during initial study period, when compared with previously established normal values (upper limit = 5.0% of dose/48 hr collection period)(3). One patient had normal fecal radioactivity (HC), and in another the value was borderline (JP). Fecal collections obtained during second study period were incomplete in 4 patients (AP, CL, JP and JD). Significant reduction of fecal radioactivity was observed in the remaining 8 patients, during the second study period (mean difference = $10.3 \pm 2.3\%$ of dose/comparable collection period, $p = <0.01$). Five of the 7 patients who showed initial abnormalities had normal fecal samples when lipid was fed 30 minutes prior to tagged triolein.

Discussion. It is apparent from the fecal radioactivity results that 10 of the 12 patients studied exhibited impaired digestion or absorption of a "standard" dose of I^{131} labeled triolein. This abnormality was either partially or completely corrected by oral ingestion of fat 30 minutes before administration of a "standard" dose of I^{131} labeled triolein. These data support the concept that the defect in such patients is primarily if not completely digestive in origin(1,4,5). Indeed, the reported data suggest that the major digestive disturbance is one of suboptimal mixing of the labeled triglyceride bolus with bile and pancreatic secretions, since optimal hormonal stimulus with a subsequent physiologic response of gall bladder and pancreas is suggested by the normal fecal values obtained with lipid pre-feeding. Although an overall

TABLE I. Effect of Pre-feeding of Fat on Blood and Fecal Radioactivity in Subtotal Gastrectomy Patients.

Patient	Study*	Blood values (% of dose/l) 1 hr	2 hr	3 hr	4 hr	5 hr	6 hr	Fecal values (% of dose)
RT	1	.71	.87	.95	1.13	1.21	1.62	16.6/48†
	2	1.11	2.14	2.66	2.61	2.36	1.95	6.0/48
AP	1	.70	1.00	1.14	1.21	1.24	1.20	36.0/48
	2	.52	1.28	2.25	3.05	3.55	2.82	.4/24
WH	1	.27	.65	1.63	2.24	2.67	2.58	10.0/48
	2	.14	.82	1.80	2.67	3.45	3.29	1.0/72
RD	1		3.13		2.90	2.51	2.26	15.6/48
	2		1.74		4.43	3.69	3.31	3.5/72
CL	1	.62	.89	1.48	2.00	1.75	1.20	27.0/24
	2	.78	1.73	2.30	3.28	3.30	2.72	.2/24
JP	1	.84	1.62	1.65	1.71	1.83	1.74	5.0/72
	2	.77	1.22	1.98	2.14	2.24	2.16	‡
ER	1	.17	.26	.26	.29	.42	.54	23.3/72
	2	.33	1.12	1.50	2.40	2.46	2.08	2.9/72
GM	1	.95	1.50	1.67	1.55	2.02	1.48	14.8/48
	2	.96	1.97	2.85	2.51	2.24	2.10	1.5/72
CD	1	.74	1.28	1.70	1.65	1.67	1.59	14.7/72
	2	.85	2.17	3.63	2.98	3.02	3.00	.4/72
JD	1	.43	.58	1.58	1.60	1.74	1.56	21.4/72
	2	.94			2.35	2.34	1.60	‡
HC	1	1.33	1.27	1.91	1.23	1.63	1.93	2.8/72
	2	1.23	2.74	3.13	2.72	2.61	2.27	.3/72
WD	1	1.52	2.50	2.65	2.51	2.51	2.30	8.3/48
	2	2.32	2.37	2.56	2.36	2.20	2.23	6.8/48

* Study 1: I^{131} labeled triolein. Study 2: I^{131} labeled triolein preceded 30 min. by peanut oil.
† Collection period (hr).
‡ Incomplete; contaminated with urine.

decrease in intestinal transport time is not present in most patients with subtotal gastrectomy(6), the bolus may enter the lower small intestine within 15 minutes after ingestion(7). In doing so, it rapidly traverses the intestinal level where maximum lipid absorption may normally occur(8). From these observations and the reported data, one may postulate a mechanism to explain the correction of abnormal fecal radioactivity when lipid was fed 30 minutes prior to administration of tagged triolein to patients with subtotal gastrectomy (Billroth II): 1. Pre-fed lipid stimulates hormonal secretion with resultant physiologic flow of bile and pancreatic juices. 2. Bile and pancreatic juices are available for mixing with the tagged lipid bolus at an intestinal site where lipids are maximally absorbed(5). Since the intestinal hormones are characterized by a short period of latency and little persistence of action(9), the timing of the "priming" dose may be variable in individual patients, which might explain the fact that fecal radioactivity was only partially corrected in 2 patients.

Summary. The I^{131} tagged triolein test was performed in 12 patients with subtotal gastrectomy (Billroth II) in the fasting state and 30 minutes after oral ingestion of a fat meal. In the fasting state, increased fecal radioactivity was observed in 10 patients. When lipid was pre-fed, there was a significant decrease in radioactive content of stool and increase in blood radioactivity. The data support the concept that the defect exhibited by these patients is primarily digestive and suggest that the major disturbance is one of inadequate mixing of labeled triglyceride with the upper intestinal contents necessary for proper lipid digestion.

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Glucose as Adjunct to 3.3-Dimethyl-1-Phenyltriazene in its Action upon Sarcoma 180. (24248)

CHARLES D. STEVENS, J. A. MUHLENPOH, MALCOLM E. TURNER, KLAUS STEMMER, I. RUCHMAN AND CHARLES J. FELDHAKE

Kettering Laboratory in Dept. of Preventive Medicine and Industrial Health, College of Medicine, University of Cincinnati, and Scientific Labs., Wm. S. Merrell Co., Cincinnati, O.

The characteristic acidity of cells of malignant tumors may make them more susceptible than normal cells to damaging, acid-sensitive chemicals(1,2). One such chemical is 3,3-dimethyl-1-phenyltriazene (PT). It inhibits Sarcoma 180 and other tumors(1,3), perhaps by releasing diazonium ion in a reaction catalyzed by acid(1). To learn whether the increased acidity of tumors might alter the action of PT on Sarcoma 180 in mice it was proposed to inject glucose prior to each day's injection of PT. The administration of glucose is known to increase acidity of tumors (4). This report presents results of such an experiment, together with ancillary observations.

Materials and methods. PT was prepared by the method of Elks and Hey(5). It was characterized by boiling range and absorption spectra in 3 solvents. It distilled at 100-101°C (uncorr.) and 5 to 5.5 mm Hg pressure. Common logarithms of molar extinction coefficients at 225, 285 and 308 mμ were, for PT in 95% ethanol, 3.93, 4.08 and 4.06, respectively. LeFevre and Liddicot(6) reported 3.97, 4.14 and 4.11 as the corresponding values. In iso-octane the logarithms were 3.99, 4.12 and 4.06 at the same wave lengths. In water they were 3.91, 4.10 and 4.10, and a maximum value of 4.12 was obtained at 302 mμ. No comparable data are available. Ultraviolet measurements were made with 1 cm cells and a Beckman DK spectrophotometer. The effect of PT upon tumor growth was measured in female albino mice weighing 17 to 20 g. Each was given by trocar a subcutaneous implant of Sarcoma 180 weighing 7.5 mg average. Injections of PT were begun one day after implantation and continued once daily for 5 days (7 days in preliminary assay). PT was dissolved in peanut oil (1 g/100 ml) and given intraperitoneally. Some of these mice were also injected subcutaneously with glucose in a solution containing 20 g/100 ml in 0.85% sodium chloride. They were given 0.8 ml/mouse/day (8 g glucose/kg) always one hour before PT. On 8th day after implantation of tumor, the mice were again weighed and the external diameters of their tumors were measured. The tumors were then excised, weighed, fixed in formalin, imbedded in paraffin and stained with hematoxylin-eosin for microscopic examination.

Results. The results of a preliminary assay and of the main experiment are presented in Table I. A difference in degree of effect of PT between preliminary assay and main experiment is unexplained. A similar variability is to be found in the data of Clarke *et al.* (1).

To analyze data from the main experiment, that with glucose, measurements of tumor weight and body weight, summarized in Table I, were transformed into a new metric of response by taking the common logarithm of the ratio of each tumor weight to the difference between initial and final body weight after adding 10 g to the difference to make

TABLE I. Effects of PT in Oil and of Glucose, Separately and in Combination, upon Growth of S-180 in Mice.

No. of mice	Daily treatment	Avg body wt (g)		Tumor wt (mg)		Tumor diameters (mm)
		Initial	Final	Avg	Range	
		Preliminary assay				
5	50 mg PT/kg			222	126–317	8.7
5	25 mg "			348	334–354	9.9
5	12.5 mg "			406	217–645	10.5
5	No treatment			455	274–578	10.1
		Main experiment				
10	50 mg PT/kg + .8 ml glucose	18.0	13.2	51	21– 94	5.3
10	50 mg PT/kg	18.0	13.2	79	31–122	5.6
10	.8 ml glucose + .1 ml oil	18.2	18.9	450	297–734	10.4
10	No treatment	18.8	19.6	481	195–835	10.5

it always positive. Transformation was used to produce an approximately additive metric of response adjusted for any general change in body weight. Thus effects on both tumor and body weights were included in a single numerical evaluation; the common alternative is to list them separately. Total variation in this new metric of response was analysed into 4 parts: one part due to the effect of glucose, another to PT, a third to interaction of glucose and PT, and a residual due to uncontrolled sources of variation. There was found a probability of about 7 in 8 that injections of glucose and peanut oil *per se* had an effect on tumor weight. Inhibition of another tumor by glucose has been observed by Bass and Feigelson(7). PT as expected had a great effect. Alteration of the effect of PT by glucose was slight, with a probability of about 1 in 5 that the observed alteration was due to chance alone.

Microscopic examination of neoplasms from mice injected with PT revealed little difference in cellular structure of those from mice which had also received glucose, as compared with those from mice that had not; the latter showed more necrosis and less differentiation of cellular elements. Neoplasms from mice not given PT exhibited greater pleomorphism and mitotic activity, together with more frequent areas of necrosis. No appreciable effects of injections of glucose and oil without PT were seen.

Discussion. That the effectiveness of glucose in altering the action of PT was not greater might be explained in many ways. For example, perhaps glucose injections produced only slight increase in tumor acidity. This could have been because the tumors were somewhat acid initially, or alternatively because acid failed to accumulate in them.

An initially acidic condition could have been caused by prolonged hyperglycemia due to PT. The only other triazene tested for effect on blood sugar, N-p-chlorophenyldiazothiourea, has been shown to produce prolonged hyperglycemia in rats(8). Hyperglycemia was looked for following injection of PT into each of 6 mice with tumors and 4 mice without. On the day following tumor implantation, tail blood was taken, either PT in peanut oil or the oil alone was injected intraperitoneally, and blood was again taken at 1 and at 2 hours after injection. This procedure was repeated with the same mice on the 2 following days; mice without tumors were tested only once. Samples of 0.020 ml of blood were obtained, and sugar was measured by Nelson's method(9). Analysis of total variation in these data into 18 parts, each associated with the various conditions of the experiment, revealed no source of variation with as much as a 10 to 1 probability of being the result of anything but chance. Almost inappreciable alteration of blood sugar by PT, about 2 mg/100 ml of blood, was found.

Another explanation of the ineffectiveness of glucose might be that absorption of PT was too slow to permit its reaching the tumor at

TABLE II. Recovery of PT and of Peanut Oil from Abdominal Cavities of Mice following Intraperitoneal Injection, with and without Injection of Glucose, One Hour Prior to Injection of PT in Oil.

Time from inj. of PT to killing (min.)	% of inj. PT recovered	% of inj. oil recovered
Without inj. of glucose		
1	61	94
2	68	93
6	50	79
60	4	63
60	6	77
60	15	68
60	19	31
61	2	56
With subcut. inj. of glucose		
35	24	75
38	26	30
77	4	62
92	3	70
120	2	44
120	5	12
With intraper. inj. of glucose		
59	71	101
60	30	88
60	72	100
61	12	91

the time tumor acidity was increased. Because no facts concerning the fate of PT in the body were known, a simple analytical technic based upon ultraviolet spectra was originated. With it, rate of disappearance of the 50 mg/kg dose of PT in peanut oil from the abdominal cavity was measured after its intraperitoneal injection. The mice were killed by withdrawal of blood from the heart, or by evacuating the air from a chamber in which they were placed. The abdominal cavity was rinsed with 40 ml of iso-octane followed by 2 ml of 1.2% aqueous sodium bicarbonate solution. The washings were shaken in a bottle for 10 minutes in darkness to insure extraction. The optical densities of the iso-octane solutions at 302 and at 240 mμ were measured. From these measurements the recovery of PT and of peanut oil, respectively, were calculated. Similar extracts from uninjected mice showed negligible optical densities (less than 0.03) at these wave lengths.

The data (Table II) indicate rapid disappearance of PT and slow disappearance of oil (the estimated half time was 60 minutes) from the abdominal cavities of mice other than those injected intraperitoneally with glucose. As to the latter, the disappearance of both PT and oil was slow and variable. Because of both these findings, intraperitoneal glucose injections were not used in the main experiment. Blood samples of 1 ml contained too little PT for measurement by the technic noted in the preceding paragraph.

Microscopic observation, using a simple, possibly novel, histochemical technic, confirmed the supposition that intact PT may reach tumor cells. Appearance of PT in appreciable concentrations in liver and, a little later, in tumors, was demonstrated within 15 to 30 minutes after intraperitoneal injection of doses of 200 to 500 mg PT in oil/kg. This was done by treating frozen sections with 1% aqueous N-(1-naphthyl)-ethylenediamine dihydrochloride and *N* hydrochloric acid. Triazene promptly hydrolyzed and the diazonium ion then coupled to yield dyes. These stained the nuclei purple and the cytoplasm orange with cherry-red granules. Squamous epithelium of skin treated in the same way stained similarly. These experiments are the only known demonstration of the catalysis of triazene decomposition by acid in tissues.

Summary. The effects of intraperitoneal injections of 3,3-dimethyl-1-phenyltriazene dissolved in peanut oil and of subcutaneous injections of glucose, separately and in combination, upon growth rate of Sarcoma 180 in mice were measured. The triazene markedly depressed tumor growth. This effect was only slightly altered by injections of glucose. Glucose itself had a slight effect upon tumor growth. The triazene disappeared rapidly from the abdominal cavity. By acidification and coupling with N-(1-naphthyl)-ethylenediamine, it could be demonstrated in tissue. Triazene injections were without effect on blood sugar.

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Copper Metabolism. XXVIII. Influence of Biliary Duct Ligation on Serum and Tissue Copper.* (24249)

H. DEMPSEY,† G. E. CARTWRIGHT AND M. M. WINTROBE
Department of Medicine, University of Utah College of Medicine, Salt Lake City

In both man(1,2) and dogs(3), the most important excretory route for copper is through the biliary system. However, no detailed studies have been performed in animals to determine the influence of ligation of bile ducts on tissue copper. The purpose of this paper is to present the results of studies on tissue and serum copper in rats with ligated bile ducts. Although normal serum copper values have been observed in swine and dogs with obstruction to outflow of bile(3), hypercupremia has been observed in patients with biliary cirrhosis(4). Copper accumulates in livers of patients with cirrhosis of the liver and associated cholangiolitis, but not in livers of most patients with uncomplicated cirrhosis (5). In one patient with primary biliary cirrhosis, an amount of copper (426 mg) was found in the liver, which was in excess even of that usually observed in livers of patients with Wilson's disease(5). Several investigators(2,6) have observed that a smaller proportion of intravenously administered radioactive copper is excreted in stools of patients with Wilson's disease than in stools of normal subjects. It has been suggested that the excessive deposition of copper in tissues may be due to impaired biliary excretion of copper (6). Therefore, it seemed of interest to determine if impairment of the outflow of bile in animals would be accompanied by excessive deposition of copper in the liver, brain and kidneys.

Methods. Adult male rats of Sprague-Dawley strain, 150 to 200 g in weight, were used. The animals were grouped into units of 3 of the same weight. One animal in each unit, designated as "bile duct-ligated," was anesthetized with sodium "Nembutal" solution given intraperitoneally. The common bile duct was located through a paramedian incision, ligated in 2 places, and sectioned between the ligatures. A careful search was made for accessory ducts. When these were found, they were also ligated and sectioned. The second animal in each unit, designated as "sham-operated," was treated in a similar manner except that the bile duct was not ligated or sectioned. The third animal in each unit, designated as "control," served as a dietary control. Bile duct ligation impaired the appetite of rats. To maintain dietary intake of copper at as constant a level as possible, the "bile duct-ligated" animal in each unit was offered 25 g of diet daily. On the following day, the uneaten portion of the diet was weighed and an amount equal to that consumed by the rat was fed to the "sham-operated" and "control" animal of that unit. Composition of diet was as follows (g/kg): casein, 200; sucrose, 640; lard, 110; sodium chloride, 6.75; magnesium carbonate, 4.25; monopotassium phosphate, 2.85; calcium phosphate, 23.15; potassium chloride, 3.35; potassium iodide, 0.1135 calcium carbonate, 8.45; ferric pyrophosphate, 1.05; copper sulfate, 0.011; manganese chloride, 0.01; zinc oxide, 0.0008; cobalt chloride, 0.008; and choline

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TABLE I. Mean Values for Serum, Liver, Kidney and Brain Copper in "Control," "Bile Duct-Ligated" and "Sham-Operated" Rats.

Group	Length of observation, wk	No. of animals	Serum copper, μg/100 ml	Liver copper	Kidney copper	Brain copper
				μg/total organ		
Control	2 & 4	25	118 ± 27	28.0 ± 10.0	12.1 ± 5.7	4.2 ±1.14
Sham-operated	2	12	128 ± 25	29.0 ± 10.4	8.5 ± 2.0	4.6 ± .32
Idem	4	12	143 ± 31	37.0 ± 9.6	15.0 ± 6.0	4.1 ± .64
Bile duct-ligated	2	14	195*	23.3 ± 10.2	9.0 ± 6.2	4.0 ± .43
Idem	4	11	273 ±100	82.0 ± 29.2	29.0 ± 7.5	4.3 ± .22

Figures refer to mean ± one stand. dev. * Mean of 7 sera which were pooled.

chloride, 2.3. Vitamins were added to the diet as follows (mg/kg): thiamine hydrochloride, 12.5; riboflavin, 6; nicotinic acid, 60; pyridoxine hydrochloride, 10; calcium pantothenate, 25; inositol, 5; para-amino benzoic acid, 5; pteroylglutamic acid, 15; Vit. E, 7.6; and Vit. K, 7.6. In addition, 23,000 units of Vit. A and 4250 units of Vit. D were added to 1 kg of diet. Following successful ligation of bile ducts, progressively deepening icterus of the ears, nose, and skin of animals was noted within 4 days. Animals which did not show a significant degree of jaundice in this period of time were excluded. Severely ill animals together with their controls were sacrificed during the 2nd week of the experiment. The remainder of the animals were killed during the fourth week. The animals were anesthetized with ether and exsanguinated *via* the abdominal aorta. The liver, kidneys and brain were removed, rinsed with copper-free water, weighed and stored in the frozen state. Methods for estimation of serum(7) and tissue copper(5) have been described previously.

Results. The results are summarized in Table I. Values for serum, liver, kidney and brain copper in the "control" group 2 weeks after beginning of experiment were the same as after 4 weeks. Therefore, all of animals in the "control" group, regardless of time of sacrifice, were analyzed as one group.

Serum copper values in the "bile duct-ligated" group increased significantly after 2 weeks as compared with "control" and "sham-operated" groups. However, liver, kidney and brain copper values were not significantly different at this time from mean values in the 2 other groups. ($P = 0.1$). Nevertheless, by the fourth week, the amount of copper in liver and kidney was markedly increased as compared with both "control" and "sham-operated" rats ($P = <0.01$). At no time was a significant increase in brain copper observed.

Discussion. Under conditions of these experiments, obstruction to the outflow of bile was associated with excessive deposition of copper in serum, liver, and kidneys. Since the biliary system is the main excretory route for copper(1-3), the most logical explanation for these changes is that copper was retained because excretion of this element was impaired. It has been shown in dogs that when excretion of copper *via* the bile is impaired, excretion of copper by the kidneys increases (3). Therefore, it is not surprising that deposition of copper occurred in the kidneys.

In rats and man(4,5), but not in dogs and swine(3), obstruction to outflow of bile is associated with hypercupremia. The reason for these differences is not apparent. It would be helpful to know if hypercupremia in rats and man is due to increase in ceruloplasmin or due to increase in direct-reacting fraction of serum copper(8). Unfortunately, the direct-reacting copper fraction cannot be measured in the presence of jaundice.

It is interesting that although copper was deposited in liver and kidneys of rats with ligated bile ducts, no deposition of copper was observed in the brain. Thus, distribution of deposited copper differed from that in Wilson's disease(9). It would have been desirable to maintain animals for a longer period of time with the bile ducts ligated. However, this was not possible since the animals would not have survived under these conditions for more than one month.

It has been suggested that excessive deposition of copper in tissues of patients with Wil-

son's disease is due to formation of abnormal proteins in tissues which have a high avidity for copper(10,11). Such a mechanism does not appear to be necessary for excessive deposition of copper in the liver and kidneys of animals with bile ducts ligated or in animals fed a high copper diet(12).

Summary. Ligation of the bile ducts of adult male rats was followed by hypercupremia and deposition of copper in liver and kidneys, but not in the brain.

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Relation Between Structure of Sulfonylurea Compounds and their Effect on Frog Muscle Metabolism.* (24250)

D. R. H. GOURLEY

Department of Pharmacology, University of Virginia Medical School, Charlottesville

Two sulfonylurea compounds, carbutamide and tolbutamide,† have been studied extensively as hypoglycemic agents. Although in normal dogs, rats and toads the 2 compounds are equally effective in reducing concentration of glucose in blood(1), they differ markedly from each other and from insulin in their effect on *in vitro* metabolism of intact frog muscle(2). Carbutamide (R = NH_2 in Fig. 1) inhibits oxygen consumption of frog muscle to a small but significant degree, as does the same concentration of sulfanilamide. Tolbutamide (R = CH_3 in Fig. 1), on the other hand, greatly stimulates oxygen consumption of muscle due to increased production of lactate from muscle glycogen. The fact that these compounds differ only in part of molecule labeled R in Fig. 1, suggested that the difference in effect on muscle was due to the type of substitution on the R position of the molecule. To investigate this possibility, similar experiments were done with a series of sulfonylbutylurea compounds which differ only in the R position in Fig. 1.

Methods. Oxygen consumption of intact

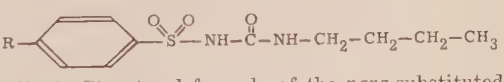

FIG. 1. Structural formula of the *para*-substituted sulfonylbutylurea compounds.

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† Tolbutamide (1-butyl-3-*p*-tolylsulfonylurea) was supplied by Upjohn Co. through the courtesy of Dr. C. J. O'Donovan; carbutamide (1-butyl-3-sulfanilylurea) was supplied by Lilly Research Labs. through the courtesy of Dr. W. R. Kirtley; 1-butyl-3-*p*-carboxyphenylsulfonylurea was a gift of Dr. L. H. Louis, Univ. of Michigan; all other sulfonylbutylurea compounds were supplied by Farbwerke Hoechst Ag. (Germany) through the courtesy of Dr. W. Meier.

TABLE I. Effect of Sulfonylbutylurea Compounds with Different Substitutions in the *para* Position of the Ring on Oxygen Consumption of Frog Muscle *in Vitro*.

Substitution	Conc., mM	No. of muscle pairs	O_2 consumption, μM/g/hr Control	Exp.	Difference, %
NH_2-	7.5	12	1.18 ± .03	1.08 ± .04	− 8*
Nothing	7.5	6	1.18 ± .05	1.35 ± .08	+ 14*
CH_3-O-	7.5	6	1.01 ± .08	1.22 ± .07	+ 21*
COOH-	7.5	9	1.39 ± .11	1.42 ± .05	+ 2
CH_3-	7.5	12	1.33 ± .06	3.75 ± .34	+182†
	10	15	1.31 ± .04	6.65 ± .32	+408†
CH_3-CH_2-	2.25	6	1.15 ± .06	5.15 ± .69	+348†
$(CH_3)_2$-CH-	.90	6	1.19 ± .08	3.97 ± .34	+234†
$(CH_3)_3$-C-	.54	6	1.26 ± .07	4.56 ± .20	+262†

* Significant at 5% level. † Significant at 0.1% level.

muscles dissected from legs of the Leopard frog, *Rana pipiens* Schreber, was measured in a Warburg respirometer at 20.0 ± 0.02°C over 6 hours as described previously(3). The iliofibularis and tibialis anticus longus muscles from one leg were placed together in Ringer's solution to serve as controls while the 2 corresponding muscles from the opposite leg of same animal were placed in Ringer's solution in which the compound under study was dissolved as sodium salt.

Results. Increased rate of oxygen consumption caused by tolbutamide is considered from previous work(2) to be due to oxidation of excess lactate. The lactate appearing in muscle and later in medium results from increased breakdown of muscle glycogen. Muscle glycogen and concentration of lactate in the medium at end of incubation period were also determined routinely, and increased oxygen consumption coincided with decreased glycogen and increased lactate in the muscle. Since measurement of oxygen consumption rate provides an accurate and sensitive measurement of the primary effect of the compounds, only these data are presented.

The results are summarized in Table I in which average hourly rate of oxygen consumption is given in μmoles/g wet weight of tissue. The molecular group substituted in the R position of the sulfonylbutylurea molecule in Fig. 1 is listed in the first column. The data obtained previously(2) with carbutamide (R = NH_2) and tolbutamide (R = CH_3) are included for comparison. At a concentration of 7.5 mM tolbutamide rate of oxygen consumption rose within 3 hours to an average of 182% of control level, and this new rate was maintained for the remainder of the 6-hour incubation period. In 10 mM tolbutamide oxygen consumption stimulation was much greater but concentrations of tolbutamide higher than 10 mM were deleterious to muscles, and the increased rates of oxygen consumption were not maintained throughout period of observation. Therefore, comparison of effects of different compounds on oxygen consumption was made at concentrations of 7.5 mM or less.

The sulfonylbutylurea molecule with no substitution at position R increased oxygen consumption rate only slightly compared with tolbutamide at the same concentration. Similarly, when a methyl group is connected to the molecule through oxygen (R = CH_3-O) instead of directly as in tolbutamide, or when a carboxyl group is connected to the molecule (R = COOH), the effect on oxygen consumption is relatively small or absent. The negative result with the carboxyl analog (1-butyl-3-*p*-carboxyphenylsulfonylurea) is of interest because Louis, Fajan, Conn, Struck, Wright and Johnson(4) found that this compound is the principal metabolic product formed when tolbutamide is administered to humans. The carboxyl analog does not produce hypoglycemia in dogs, rats or humans. However it cannot be concluded that the effect of tolbutamide on frog muscle metabolism is related to its hypoglycemic effect *in vivo*, since carbutamide had no effect on frog muscle glycogen (by direct analysis) although it, too, is an effective hypoglycemic agent.

The compound with an ethyl group in the

R position of Fig. 1 is very effective in stimulating oxygen consumption. In fact, to avoid deleterious effects to the muscle, the concentration of this compound had to be lowered to 2.25 mM. The effectiveness of the molecule increased when the substituted group was changed to either isopropyl or tertiary butyl and the concentrations of these compounds also had to be decreased to avoid deleterious effects to the muscle. By making rough approximations from the data in Table I the relative potencies of the 4 effective substitutions on the sulfonylbutylurea molecule are estimated to be: methyl, 1; ethyl, 4; isopropyl, 8; tertiary butyl, 16. The relative effect of these compounds on blood glucose level of frogs is not known.

It has been known for many years that caffeine, like tolbutamide, stimulates glycolysis in frog muscle with consequent lactate accumulation and extra oxygen consumption(5, 6). Fenn(7) concluded that oxygen consumption stimulation was correlated with contracture of the muscle caused by caffeine. To determine whether tolbutamide is a contracture-producing agent like caffeine, experiments were done in which a tibialis anticus muscle was suspended in 7.5 mM tolbutamide-Ringer's solution from a carefully balanced heart lever to magnify any change in length of muscle. The opposite muscle from the same frog was similarly suspended in Ringer's solution. Muscle length was recorded at beginning of immersion and at intervals for 6 hours and no change in length was observed in either solution. This simple experiment strongly suggests that the metabolic activity initiated by the effective sulfonylbutylurea compounds occurs without mechanical change in muscle, and in this respect their action differs from that of caffeine. There is evidence that increased metabolic activity of frog muscle in a concentration of 10 mM potassium also occurs without accompanying contracture(8).

It is not known whether the effect of the caffeine molecule on oxygen consumption of frog muscle is related to addition of the methyl groups to the xanthine nucleus. However, recalculation of Gemmill's data(9) on the effect of 1-substituted theobromine derivatives on oxygen consumption of rat diaphragm muscle indicates that, in general, as the carbon chain attached to theobromine increased, the molar concentration required to stimulate the oxygen consumption by about 30% decreased. Thus it appears that addition of methyl groups in a chain to theobromine also increases the effectiveness of this compound in stimulating muscle metabolism.

Summary. The effect of sulfonylbutylurea derivatives on frog muscle metabolism depends upon the molecular group substituted on the benzene ring *para* to the main chain of the molecule. Substitution of an amino group produces an inhibitory compound; absence of any group or substitution of either a methoxy or a carboxyl radical produces a compound with little or no effect. Substitution of a methyl, ethyl, isopropyl or tertiary butyl radical produces compounds that are increasingly effective in stimulating glycolysis and oxygen consumption. These effects are similar to those caused in frog muscle by caffeine although, unlike caffeine, the sulfonylbutylurea derivatives apparently exert their effects without causing contracture.

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Influence of Endocrine Glands on Radiation-Induced Hyperpigmentation in Mice.* (24251)

Walter C. Quevedo, Jr.† and Douglas E. Smith
Division of Biological and Medical Research, Argonne National Laboratory, Lemont, Ill.

When pigmented mice are exposed to gamma radiation, the skin of extremities becomes progressively darkened(1). This hyperpigmentation results from increased deposition of melanin within the epidermis by epidermal melanocytes(1,2,3). Although hormones are known to play a role in regulation of mammalian pigmentation(4,5), the extent to which radiation-induced darkening may be dependent on their action is not known. The present study was designed to test the possibility of an endocrine-radiation interaction during hyperpigmentation. The hormonal balance of mice was varied either by surgical removal of specific endocrine glands prior to irradiation or by introduction of supplementary doses of hormones into intact animals concurrent with irradiation.

Materials and methods. C57BL (black) and LAF_1 (brown) mice 3 to 6 months old of both sexes were used. All mice were exposed for the duration of life to gamma rays from a Co^{60} source(6) and received total body doses of either 97 r/day or 125 r/day (measured in air without scatter). Pigmentation changes were studied in plantar surfaces of hind feet. An arbitrary grading system was used to measure pigment increase; grades ranged from 0, for no signs of darkening, to +++, the maximum grade, characterized by intense darkening of foot pads and by darkly pigmented bands on phalanges. Development of radiation-induced hyperpigmentation was studied in 5 groups of mice (approximately equally divided as to sex and strain) treated as follows: I. 50 mice, intact, irradiated (controls). II. 33 mice, castrated 3 weeks to 3 months prior to exposure to radiation. III. 20 mice, adrenalectomized 1 to 3 days prior to irradiation and maintained on 0.9% aqueous solution of NaCl following removal of adrenal glands. IV. 54 mice, given intramuscular injections of cortisone acetate (Merck) daily (0.625 mg/day to 6.0 mg/day), beginning with first day of irradiation. V. 24 mice, given subcut. injections of ACTH (Corticotropin-gel, Wilson) daily (1 to 4 U.S.P. units/day), beginning with first day of irradiation. Two additional experiments were performed to test for other possible endocrine-radiation interactions during hyperpigmentation. Six NIH Black rats of both sexes were hypophysectomized at 21 to 30 days of age. One to 2 weeks later, the animals were exposed to 200 r of 100-kvp X-rays, rats being lead-shielded so that only the right hind feet were irradiated. Thereafter they were irradiated in the same manner once a week for 7 weeks. Four intact controls were similarly exposed. Twelve mice of both strains and sexes were castrated 3 weeks prior to irradiation and exposed on plantar surfaces of feet to UV (G. E. Sunlamp: 110-125 V, 275 W, 60 Cycle, A.C.) for 2 minutes and 15 seconds on each of 16 consecutive days. Specimens of skin were obtained from feet of representative animals of all groups. These were fixed in a modified Tellyesniczky's solution. Paraffin sections 8 to 10 μ thick were made following routine histological procedures. Sections were stained with 2% aqueous solution of alum carmine. A number of whole-mount preparations of skin, prepared by the method of Smith and Lewis(7), were also examined.

Results. In control mice exposed to 97 r/day or 125 r/day of γ-rays, regardless of strain or sex, definite hyperpigmentation (+ grade) was observed in plantar surfaces of feet by ninth day of exposure; a ++ grade was evident by eleventh day and +++ grade between 19th and 25th day. Both castration

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† This work was performed while Dr. Quevedo was a Postdoctoral Resident Research Associate at Argonne National Lab. Present address: Biological Station, Roswell Park Memorial Inst., Springville, N. Y.

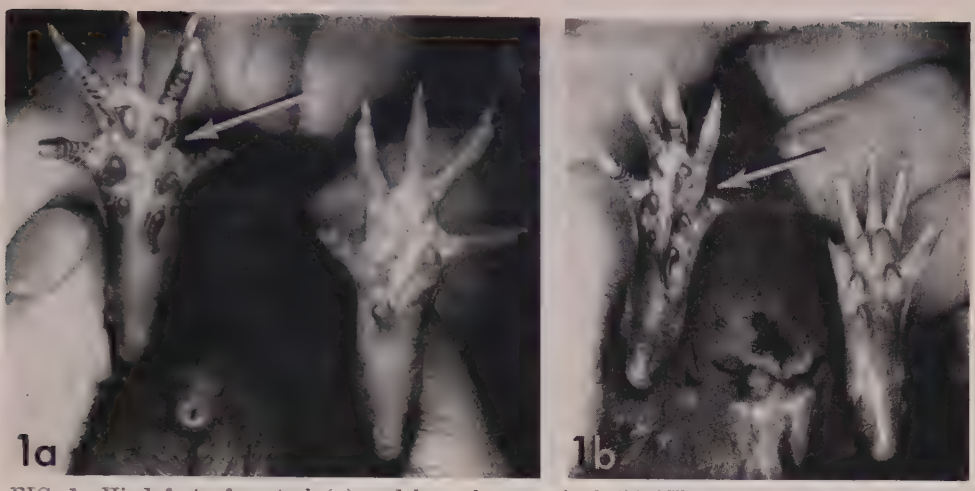

FIG. 1. Hind feet of control (a) and hypophysectomized (b) NIH Black rats after partial-body x-irradiation (200 r/wk) for 7 wk. Note intense hyperpigmentation of irradiated feet (arrows) and absence of darkening in shielded feet.

and adrenalectomy failed to modify development of radiation-induced hyperpigmentation. Similarly, irradiated mice treated with ACTH darkened at the same rate as controls. In hypophysectomzed rats, hyperpigmentation was observed in the irradiated feet by second week of exposure and increased in intensity thereafter. No obvious differences in intensity of coloration were apparent between irradiated feet of hypophysectomized and control rats (Fig. 1). The nonirradiated appendages of all animals failed to darken (Fig. 1).

In contrast, daily injections of cortisone acetate produced a striking suppression of darkening in intact, irradiated mice. In LAF_1 mice, doses of from 2.5 to 6.0 mg/day of cortisone produced the greatest inhibition of hyperpigmentation (Table I). The minimum daily dose of cortisone (0.625 mg/day) produced little to no inhibition of pigmentation. Although LAF_1 mice receiving 3 mg/day of cortisone showed no pigment increase (Table

TABLE I. Hyperpigmentation in Feet of Gamma Irradiated LAF_1 Mice Treated Daily with Cortisone.

Cortisone, mg/day	Grades of pigmentation	Duration of exposure (days) 0–8	9–10	11–15	16–18
2.5–6.0	0	100 (21)	100 (7)	100 (5)	No surv.
	+	0	0	0	
	++	0	0	0	
	+++	0	0	0	
1.25	0	100 (15)	60 (15)	0 (14)	0 (3)
	+	0	40	86	67
	++	0	0	14	33
	+++	0	0	0	0
.625	0	100 (6)	0 (6)	0 (3)	0 (2)
	+	0	100	33	0
	++	0	0	67	100
	+++	0	0	0	0
.000	0	100 (15)	0 (15)	0 (15)	0 (15)
	+	0	100	0	0
	++	0	0	100	100
	+++	0	0	0	0

Figures preceding parentheses indicate % of mice examined that exhibited grade of pigmentation shown. Figures within parentheses represent total No. of mice examined at each time interval. Decreasing numbers of observations with increasing time reflect death or sacrifice of mice in previous time intervals.

I), examination of whole-mount preparations of skin from these animals revealed that melanin formation had not been completely inhibited. The effects of cortisone on irradiated C57BL mice were quite similar to those observed in LAF_1 mice. Both adrenalectomy and treatment with cortisone (1.25 to 6.0 mg/day) brought about an early mortality of irradiated mice, the majority dying prior to nineteenth day of exposure. Only rarely did a control mouse die within this period.

Daily exposure of mice to UV brought about a darkening of plantar surfaces of feet. The response was identical in castrate and control mice however, a pronounced strain difference was evident. Darkening was observed in C57BL mice by sixth day of exposure and not until the eighth day in LAF_1 mice. In addition, LAF_1 mice did not surpass a pigmentation grade of + during the 16 day period, whereas C57BL mice achieved a grade of +++ by the twelfth day of exposure to UV.

Histological studies revealed that all instances of hyperpigmentation resulted from increased numbers of melanotic epidermal melanocytes and increased deposition by these cells of melanin within the epidermis. Only an occasional melanotic epidermal melanocyte was observed in skin from nonirradiated controls. Comparisons of pigment content in sections and whole-mounts of skin correlated well with pigmentation grades based on gross examination of plantar surfaces.

Discussion. The present results indicate that radiation-induced hyperpigmentation may occur in the absence of hormonal contributions by hypophysis, gonads, and adrenals. This is of interest in view of previous observations that hormones derived from these glands play an important role in regulation of mammalian pigmentation. It has been reported that melanophore-stimulating hormone (MSH) derived from the hypophysis elicits hyperpigmentation in humans(4), and that adrenocortical hormones suppress pigmentation in humans(4), rats(8) and hamsters(9), and enhance pigmentation in the guinea pig(10). It has been suggested that hormones of the adrenal cortex suppress pigmentation by inhibiting release of MSH from the hypophysis(4). In the present study, the inhibition of pigment formation observed in irradiated mice treated with cortisone might have resulted from direct action on the melanocytes or indirect action by suppressing release of essential pituitary hormones. The demonstration of abundant hyperpigmentation in irradiated hypophysectomized rats would appear to support the former interpretation. Failure of ACTH to inhibit pigment formation is surprising since ACTH promotes release of cortisone-like compounds from the adrenal cortices. Possibly the employed doses of ACTH were insufficint to promote release of amounts of cortical hormones adequate to produce an inhibition. It is also possible that rapid inactivation of ACTH(11) or contamination of ACTH preparations by other hormones(12) would account for its ineffectiveness in suppressing pigment formation. Failure of castration to influence development of hyperpigmentation in irradiated mice indicates that species differences may exist in this regard, since male and female human castrates have been reported to tan but poorly when exposed to ultraviolet light(5). No definitive explanation can be given at present for failure of LAF_1 mice to tan significantly on exposure to UV in contrast to the marked hyperpigmentation elicited by gamma irradiation. It is possible in both cases that the differences in quality of the radiations may account for the differences in results.

It is of interest to note that in preliminary experiments diethylstilbestrol suppressed radiation-induced hyperpigmentation in LAF_1 female mice as did cortisone. This finding is in contrast to previous reports of enhanced pigmentation in humans(13) and guinea pigs (10) treated with this compound. It is quite possible that the high daily dose of diethylstilbestrol (1 mg/day) employed in the present experiments, would account for this difference in response.

Our findings support the view that radiation-induced hyperpigmentation is largely a local phenomenon since pigmentation increase was restricted to regions directly exposed to radiation. In addition, removal of endocrine

glands known to influence pigmentation did not significantly alter the occurrence of hyperpigmentation in irradiated mice. However, that this process is not entirely refractory to hormones is evidenced by inhibition of melanin formation in irradiated mice treated with large doses of exogenous cortisone or of diethylstilbestrol.

Summary. C57BL and LAF_1 mice became hyperpigmented in the extremities when exposed daily to gamma radiation. This process was not significantly altered by adrenalectomy, castration or daily treatment with ACTH. Hypophysectomized black rats, X-irradiated weekly, developed hyperpigmentation at the same rate as controls. Although it would appear that induction of hyperpigmentation by radiations is largely free of endocrine control, daily treatment of gamma irradiated mice with large doses of cortisone will suppress pigment formation.

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Effect of Various Adrenal Steroids on Internal Fluid and Electrolyte Shifts of Fasted Adrenalectomized Dogs.* (24252)

W. W. SWINGLE, J. P. DAVANZO,† H. C. CROSSFIELD,† D. GLENISTER, M. OSBORNE AND G. WAGLE

Biological Laboratory, Princeton University, Princeton, N. J.

Glucocorticoids readily restore to normal the markedly disturbed fluid and electrolyte equilibria of adrenalectomized dogs exhibitings severe insufficiency. This is accomplished in animals deprived of food and water, by hormonal mobilization and shift of fluid and electrolytes from cells and certain tissues to the dehydrated extracellular (and intravascular) compartment. Experiments reported here indicated that aldosterone and DOC are incapable of relieving severe symptoms of adrenal insufficiency or of inducing internal redistribution of fluid and electrolytes in the *fasted* dog. The data suggest tentative assignment of certain functional roles for mineralo- and glucocorticoids in regulatory control of salt and water balance. The materials and methods used in studies on adrenalectomized dogs have been adequately described (1,2).

Results. Effect of 2-methyl-9α-fluorohydrocortisone, Aldosterone and Desoxycorticosterone on plasma and urine electrolytes and plasma volume of fasted adrenalectomized dogs recovering from insufficiency. A. 2-methyl-9α-fluorohydrocortisone (2-methyl FF free alcohol). Five dogs were tested and to-

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† New Jersey Heart Assn.

TABLE I. Plasma Volume and Hemoconcentration Changes Induced by Mineralo- and Glucocorticoids in Adrenalectomized Dogs Fasted 48-72 Hours during Recovery from Adrenal Insufficiency.

Dog No. & symptoms	Dose, mg/day	Body wt, kg	BP, mm Hg	Blood urea N, mg %	Hb, g %	Hmct, %	RBC 10^6, mm^3	Blood sugar, mg %	Plasma vol changes: cc/kg	Plasma vol changes: % increase or decrease
				2-Methyl FF						
(1) N†	0	22.91	110	18.1	10.50	30.3	4.34	85.5	51.5	
S	1	22.15	47	64.0	16.01	39.3	6.60	75.5	32.2	
M	1	20.79	64	33.2	13.90	37.6	5.03	93.5	39.0	
N	.5	20.11	92	26.1	11.38	35.4	4.22	94.5	50.3	+56.2
(2) N	0	15.60	120	24.8	11.82	34.7	5.33	81.5	52.3	
M	1	15.42	70	50.2	15.25	40.0	6.90	90.5	39.3	
N	1	14.50	104	24.6	13.98	37.5	5.28	100.0	52.0	+32.3
N	.5	14.10	119	22.6	11.30	33.2	4.73	107.0	49.8	
				Aldosterone						
(6) N	0	14.85	105	17.0	9.57	26.3	4.11	81.0	49.3	
S	4.0	14.29	50	124.0	14.73	38.3	5.47	85.0	36.6	−25.7
	Death 6 hr after inj. Final sample not taken.									
	Aldosterone—1st 24 hr 2-Methyl FF—48 and 72 hr									
(7) N	0	24.5	123	17.1	14.03	38.8	6.23	77.5	42.4	
S	8	23.41	65	64.5	15.43	40.5	7.01	78.0	41.5	
S	2	22.91	68	72.0	15.43	43.2	7.41	67.5	33.6	−20.7
N	8*	21.70	85	42.0	13.91	37.4	6.39	101.0	47.4	+41.0
N	7.5*	21.36	99	41.6	11.48	34.6	5.56	90.5	45.9	
	Desoxycorticosterone—1st 24 hr 2-Methyl FF—48 and 72 hr									
(8) N	0	17.92	122	14.5	14.03	42.0	6.99	76.5	55.2	
M	100	16.22	80	71.5	18.64	47.5	8.88	72.5	38.1	
S	25	15.31	60	54.3	16.68	44.4	8.41	86.5	35.6	−35.5
N	10*	14.51	88	46.5	14.55	41.6	7.41	95.5	45.9	+28.9
N	10*	13.95	102	38.4	13.75	40.3	7.15	108.0		

+ = Increase in plasma vol. — = Decrease in plasma vol.

* 2-Methyl FF inj. i.v. in 3 divided doses each 24 hr.

† N = Normal (adx but no symptoms). S = Severe insufficiency. M = Mild insufficiency.

tal dose given varied from 2.5-5 mg /dog. Two representative cases are given in Table I. Food and water deprivation lasted 48 hours and degree of adrenal insufficiency was severe in Dogs 1, 3, 4, but milder in Dogs 2 and 5 (Tables I and II). All exhibited characteristic blood changes associated with insufficiency: lowered arterial pressure and plasma volume, marked hemoconcentration, low plasma Na and Cl with elevated plasma K and high blood urea nitrogen. Following injection of 2-methyl FF these deviations from normal were corrected in spite of lack of food and water. The rise in plasma Na averaged 11.0 meq/l and range was from 5.5 to 13.5 meq/l. Plasma Cl also rose but not to the same extent, whereas plasma K declined 2.99 meq/l during recovery period (Table II). A profuse diuresis began shortly after injecting the steroid, with renal elimination of large amounts of Cl and K but much less Na. The quantity of K excreted amounted to 135 meq within 48 hours in Dog 1 (Table II). A marked increase in plasma volume occurred in all cases. Progressive decrease in arterial pressure characteristic of insufficiency, and elevation during recovery are apparently due chiefly to fall and rise in plasma volume (Table I.)

B. Aldosterone. Fourteen mg of synthetic d, l-aldosterone 21-monoacetate were available. The material was solubilized by the method previously described for other steroids(1,2), and administered i.v. in divided doses. Two adrenalectomized dogs were used when they had developed severe insufficiency (Table I). Dog 6 was injected with 4 mg of aldosterone, given in 2 doses of 2 mg over a 6 hour period. No improvement in the animal's condition was observed and death occurred 6 hours after initiating treatment. Dog 7 was then tested, using 10 mg of aldosterone

TABLE II. Effect of Mineralo- and Glucocorticoids on Plasma and Urine Electrolytes of Fasted Adrenalectomized Dogs during Recovery from Adrenal Insufficiency.

Dog No.	Total dose, mg	Plasma electrolyte changes during fast, meq/l			Total urine output during recovery period, cc	Urine electrolytes excreted during recovery, meq		
		Na	Cl	K		Na	Cl	K
		2-Methyl-9 alpha-fluorohydrocortisone						
1	2.5	+13.5	+ .7	−4.93	1175	37.46	107.52	135.45
2	2.5	+12.5	+2.8	−2.94	900	15.07	46.50	52.91
3	5.0	+12.0	+8.2	−2.42	1035	7.27	18.29	76.45
4	2.5	+11.5	+1.8	−2.87	931	2.50	35.70	68.09
5	4.0	+ 5.5	+4.2	−1.79	920	4.80	16.00	65.02
		Aldosterone—0 to 6 hr (death)						
6	4.0	− 3.0	−8.4	+4.84	0	—	—	—
		Aldosterone—0 to 24 hr						
7	10	+ 2.5	− .6	− .34	442	3.70	6.42	21.26
		2-Methyl-9 alpha-fluorohydrocortisone—24-72 hr						
	17.5	+11.8	+ .1	−1.67	811	6.55	24.29	104.01
		Desoxycorticosterone—0 to 24 hr						
8	125	+ 7.0	+2.0	−3.10	605	3.93	6.20	21.63
		2-Methyl-9 alpha-fluorohydrocortisone—24-72 hr						
	20	+ 6.0	+9.5	+ .48	1020	10.40	13.83	35.30

+ = Increase in plasma electrolytes. — = Decrease in plasma electrolytes.

i.v. in divided doses as follows: 4, 2, 2, 2 mg given 6 hours apart. The steroid failed to relieve symptoms which grew steadily worse during the 24 hours of medication. At the end of this period the dog was very weak and spastic and collapsed after removal of blood for sampling. It became necessary to inject 2-methyl FF (15.5 mg in divided doses over the ensuing 48 hours of continued starvation) to save the animal. The plasma Na rose 2.5 meq/l under aldosterone treatment but this apparent increase was presumably accounted for by a 20.7% decline in plasma volume accompanied by renal excretion of 442 cc of urine containing very small amounts of Na and Cl but larger quantities of K (Tables I, II).

Within 24 hours after injecting 2-methyl FF the dog was active, vigorous and all signs of insufficiency had disappeared. At the end of 72 hours of fasting, the arterial pressure, plasma volume and hemoconcentration were at control levels but blood urea nitrogen remained elevated (Table I). The plasma Na had risen an average of 11.8 meq/l in spite of a rise in plasma volume of 41.0% and a urine output of 811 cc containing 6.55 meq of Na and 24 and 104 meq respectively of Cl and K (Table II). This dog probably would have succumbed as did Dog 6 had it been kept on aldosterone treatment and the fast continued. Although aldosterone failed to raise arterial pressure and plasma volume, or to decrease hemoconcentration, blood urea nitrogen or otherwise improve the dog's condition, it did induce renal retention of Na and Cl and stimulate excretion of K over the 24 hour period studied (Table II). The Na retaining activity persisted over the period of observation.‡

C. Desoxycorticosterone. Tables I and II (Dog 8) present the pertinent data obtained from a case of adrenal insufficiency treated with 125 mg of DOC given i.v.; 75 mg as initial dose followed at 8 hour intervals by 25 mg. Symptoms were not alleviated by this regimen and the animal continued to fail. Blood pressure and plasma volume remained low but blood urea nitrogen fell 17 mg%, and some decrease in hemoconcentration took

‡ Since this article was offered for publication a third fasted adex dog could not be revived from insufficiency with 20 mg of aldosterone given i.v. in divided doses over 42 hours. Internal shift of fluid and electrolyte did not occur until glucocorticoid was injected.

place. Blood sugar rose 14 mg% in Dog 8 (Table I). This animal excreted 605 cc of urine during the 24 hours of DOC treatment which contained very small amounts of Na and Cl but considerable K (Table II). Plasma Na rose 7 meq/l but the rise in Na probably was not due to an internal shift of cation and water from cells, but presumably resulted from the additive effects of renal retention of Na plus a sharply reduced volume of extracellular fluid. Plasma volume diminished by 35.5% and urine output was 605 cc during the interval the Na increased in the plasma. Thus DOC behaved like aldosterone in causing retention of Na and Cl and excretion of K. There was no evidence of an internal fluid and electrolyte shift. Treatment of this animal with 2-methyl FF restored activity and vigor, increased the plasma volume, raised arterial pressure to normal and stimulated the kidneys to excrete over a liter of urine (Table II). Evidently large amounts of Na and Cl were shifted into the extracellular space since plasma Na increased 6 and the Cl 9.5 meq/l despite: (1) a rise in plasma volume of 28.9% and (2) loss of 1625 cc of fluid as urine during the 72 hour interval of food and water deprivation (Tables I, II).

Discussion. Considering the amount of fluid and electrolyte shifted into the extracellular compartment during 2-methyl FF treatment of the fasted dogs, and the lack of capability of the adrenal-insufficient animal to bring about such a shift in the absence of the correct type of corticoid, it is assumed that adrenal steroids possessing potent glucocorticoid activity function as homeostatic mechanisms in the salt and water balance of the body by enabling the animal to freely transfer fluid and electrolyte from one body compartment to another.

Aldosterone was largely, if not entirely devoid of those properties necessary to effect internal redistribution of fluids and electrolytes in adrenalectomized dogs when food and water were withheld during recovery from insufficiency. The fasted animal succumbs unless some type of corticoid containing glucocorticoid activity is administered. The activity of desoxycorticosterone did not differ essentially from that of aldosterone. Numerous investigators have shown that aldosterone markedly stimulates renal retention of Na(3-7), and also exerts some regulatory control over the quantities of this ion eliminated by such secretory structures as salivary glands(8), and probably also tear and sweat glands. Hence this natural mineralo-corticoid may be primarily concerned with regulatory control of the electrolyte pattern of the extracellular fluids. Deviations from normal in ionic content of these fluids would presumably lead to increased or decreased secretion of aldosterone(6,9,10), eventually resulting in retention or excretion of the offending ion as circumstances demanded.

Summary. Shifts of fluid and electrolytes from intra- to extracellular compartments follow i. v. injection of potent glucocorticoids in adrenalectomized dogs deprived of food and water during recovery from insufficiency. Aldosterone and desoxycorticosterone medication failed to improve symptoms, raise the lowered plasma volume or arterial pressure. Internal redistribution of water and electrolytes did not occur but renal retention of Na and Cl was evident. Possible roles for mineralo- and glucocorticoids in regulatory control of salt and water balance are discussed.

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Influence of Age on Metabolic Activity of *Mycobacterium tuberculosis*. (24253)

RICHARD H. LYON, HERMAN C. LICHSTEIN AND WENDELL H. HALL
Veterans Administration Hospital, Minneapolis, and the Department of Bacteriology and Immunology, University of Minnesota Medical School, Minneapolis

Metabolic studies of *Mycobacterium tuberculosis* have been carried out in the main using bacterial cells obtained from surface growth on a variety of media incubated for 3-5 weeks (1-3). This method has the advantage of harvesting relatively large numbers of cells from small amounts of media, but it has the disadvantage of employing tubercle bacilli which have passed the logarithmic phase of growth. With the introduction of technics allowing growth of tubercle bacilli in a submerged-dispersed condition and the availability of high-speed refrigerated centrifuges it is no longer a serious problem to harvest bacterial cells from large quantities of media. Furthermore, the more rapid rate of growth under these conditions permits use of younger bacterial cells which may not only be more active metabolically than their older counterparts grown on surface cultures but may actually differ in their metabolic pathways. Although comparatively little work has been reported relating metabolic activity of the tubercle bacillus with the age of the culture, Loebel, Shorr, and Richardson(4) demonstrated that younger cells metabolize more actively than older cells. It would appear therefore that many investigators have sacrificed metabolic activity per cell in order to obtain improved yields of cells.

The present study was initiated to investigate the metabolic activity of *M. tuberculosis* grown dispersed in liquid media and to relate the influence of age on this activity. The system chosen was oxidation of lactic acid. Intact cells were employed in order to gain a better physiological picture being fully cognizant of the fact that disrupted cells may be of value in future investigations.

Materials and methods. *M. tuberculosis* strain H37Ra* was used throughout the study and was cultured in a submerged-dispersed condition employing the medium of Sauton (5). Although a wetting agent is ordinarily not included in Sauton's medium, Tween 80† (final concentration 0.02%) was incorporated in the present study to permit dispersed growth of the tubercle bacillus. The media were dispensed in liter quantities into 2000 ml Erlenmeyer flasks and inoculated with 20 ml of a 7 day culture of H37Ra grown in the respective medium. The cultures were incubated under stationary conditions at 37°C except that the flasks were rotated briefly 3 times a day throughout the incubation period for purposes of aeration.

The bacterial cells were harvested by centrifugation, washed twice with distilled water and resuspended in the same menstruum to give a final concentration of approximately 10-12 mg dry weight of tubercle bacilli per ml. The dry weight was determined either by direct weighing of an aliquot of cell suspension after drying in an oven at 100°C for 24 hrs or by an indirect method. This consisted of interpolation from a previously constructed straight-line graph relating mg dry weight of bacterial cells per ml of washed cell suspension to % optical transmission at a wave length of 600 mμ using the Coleman Junior Spectrophotometer. Experimental results revealed that the greatest accuracy was obtained when the washed cell suspension was diluted to a point where the optical transmission fell within the range of 40-60%.

The Warburg constant volume respirometer was used to measure oxygen uptake of the resting cell suspensions. The temperature of the bath was adjusted to 37°C and the shaking rate to 140 strokes per min. The main compartment of the Warburg vessel contained phosphate buffer, bacterial cell suspension and

* Obtained from the U. S. Public Health Service, Communicable Disease Center, Chamblee, Ga.

† Polyoxyethylene sorbitan monooleate produced by the Atlas Powder Co., Wilmington, Del.

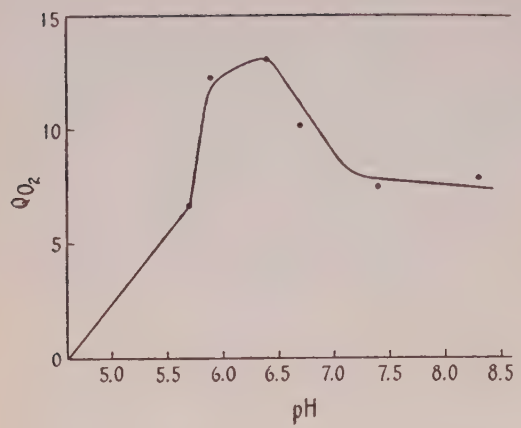

FIG. 1. Effect of pH on oxidation of lactic acid by washed cell suspensions of *M. tuberculosis* strain H37Ra. 0.066 M phosphate buffer; 0.022 M lactate; age of culture, 7 days; Sauton's medium; 3.6 mg dry wt bacterial cells/cup.

sufficient distilled water to bring the volume to 3 ml; the inner well contained 0.15 ml of 20% KOH; the side arm housed the substrate which was tipped after temperature equilibration. Readings were made at suitable intervals for a period of 5 hrs and the results are expressed in terms of Q_{O_2} *i.e.*, μl O_2 consumed per mg dry weight of cells per hr.

Results. Prior to the investigation of the influence of age of culture on metabolic activity of *M. tuberculosis* it was necessary to learn the optimal pH and the substrate concentration for such studies. The results presented in Fig. 1 relate the rate of lactate oxidation with pH. A final concentration of 0.066 *M* phosphate buffer was employed in order to achieve ample buffering capacity over the wide range of pH studied. It is quite clear from the data presented that the optimum range of pH was approximately 5.9-6.6 with maximum activity at pH 6.4. It is also manifest that the rate of lactate oxidation fell off quite markedly on either side of the optimal range.

With respect to substrate concentration the data in Fig. 2 reveal that rate of lactate oxidation increased markedly at substrate levels between 1.66 and 2.32 x 10^{-2} *M*. Concentrations of lactic acid in excess of the latter figure caused no further increase in metabolic activity.

A series of experiments were performed next, designed to relate physiological activity (using lactate oxidation as a parameter) with age of the bacillus. Aliquots of culture were removed at daily intervals and turbidimetric measurements made of the unconcentrated culture and of the culture concentrated 5 times. The latter procedure was considered advisable because of the sparse growth of the organism especially during the first week of incubation. Concentration was achieved by centrifugation of a 10 ml aliquot of culture for 30 min at 12,500 x gravity followed by resuspension of the sedimented cells into 2 ml of the supernatant fluid. Resuspension was facilitated by making use of a Teflon homogenizer. The results so obtained were employed for construction of the growth curves presented in Fig. 3. At intervals from the 4th through the 20th day of incubation, respiration studies were performed employing washed bacterial cell suspensions on the day of harvesting in order to avoid varying reductions in Q_{O_2} following refrigeration of such suspensions. The results of such studies (Fig. 3) revealed maximum lactate oxidation between the 10th and the 12th day of incubation which approximated the mid-portion of the logarithmic phase of growth.

Discussion. The results herein presented make known that the metabolic activity of *M. tuberculosis,* as revealed by a study of lactate oxidation, is influenced markedly by

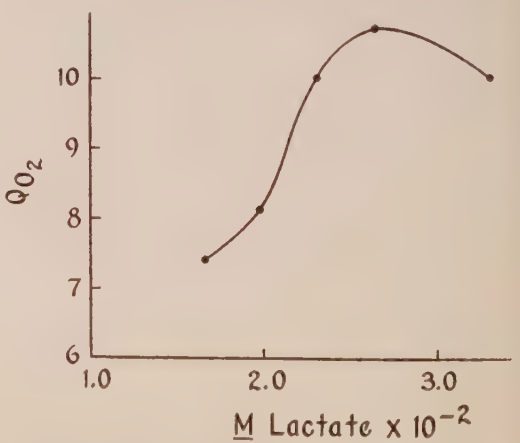

FIG. 2. Effect of substrate concentration on rate of oxidation by washed cell suspensions of *M. tuberculosis* strain H37Ra. 0.066 M phosphate buffer, pH 6.4; 7.3 mg dry wt bacterial cells/cup; age of culture, 7 days; Sauton's medium.

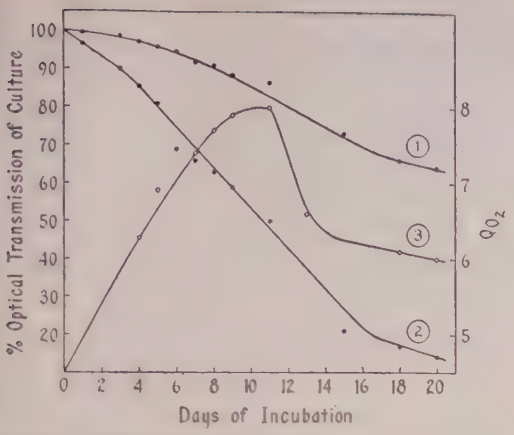

FIG. 3. Effect of age of culture on rate of lactate oxidation by washed cell suspensions of *M. tuberculosis* strain H37Ra. Curve 1 = % optical transmission of culture. Curve 2 = optical transmission of culture concentrated 5 times. Curve 3 = Q_{O_2}. 0.066 M phosphate buffer, pH 6.4; 0.022 M lactate; 6-9 mg dry wt bacterial cells/cup; Sauton's medium.

age of the bacterial cells. It is manifest from the data given that rate of lactate oxidation by washed cell suspensions increases with age of the cells, reaching a maximum between the 10th and the 12th day of incubation which coincides with the middle of the logarithmic phase of growth. Upon continued incubation, rate of lactate oxidation falls rapidly reaching a minimum during the stationary phase of the bacterial growth cycle. Thus, it is evident that the tubercle bacillus responds in this respect in a similar manner to other micro-organisms(6) and suggests that young cells derived from dispersed cultures might properly be more widely used in studies of the physiology of this organism. Although admittedly these data were obtained using only one strain of the tubercle bacillus and inspecting only one enzyme system, the results suggest further study along these lines. Furthermore, since it has been established that the lipid content of tubercle bacilli increases with age(7), which may well influence the permeability of the older intact cells, it would appear desirable to utilize the younger cells for investigative purposes.

Summary. A study of lactate oxidation by washed cells of *M. tuberculosis* strain H37Ra revealed a marked influence of the age of the cells. Cells obtained during the logarithmic phase of growth were the most active metabolically.

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Biliary Excretion of Co^{60} Labeled Vitamin B_{12} in Dogs.* (24254)

D. A. Williган,† E. P. Cronkite,‡ L. M. Meyer§ and S. L. Noto§

Medical Dept., Brookhaven Nat. Laboratory, Upton, N. Y., College of Veterinary Medicine, University of Minnesota, St. Paul, South Nassau Communities Hosp., Oceanside, N. Y.

Previous reports indicate that radioactivity is present in bile and feces after injection of labeled B_{12}, but observation periods were short and data not critically analyzed(1,2,3). The following study was instituted to evaluate the rate of Co^{60} excretion in bile and its role as a source of fecal radioactivity after intravenous

* Supported by U. S. Atomic Energy Com. and performed at Medical Dept., Brookhaven Nat. Laboratory.

† Research Collaborator at Brookhaven from College of Veterinary Medicine, University of Minnesota.

‡ Medical Dept., Brookhaven Nat. Lab.

§ South Nassau Communities Hosp.

TABLE I. Total Biliary Excretion in 5 Dogs following Intravenous Administration of Co^{60} Vit. B_{12}.

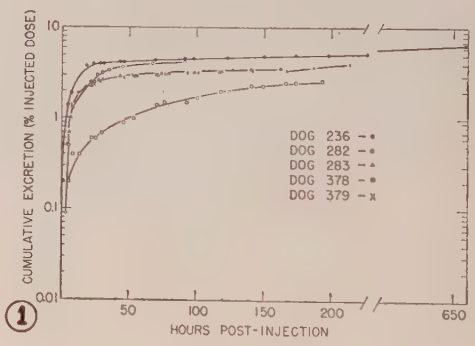
Dog No.	Sex	Wt, lb	Total dose inj., μc	Total vol bile collected, ml	Period of collection, hr	% of inj. dose in bile
282	♂	52	3.48	250	96	4.2
283	♂	38	3.48	661	162	3.6
236	♂	37	3.48	3245	658	6.6
378	♂	48	2.096	889	193	2.6
379	♂	48	2.096	1342	213	4.0

Lot V 5-6-6—20 cc vial contains 22.96 μc equivalent to 25.96 μg.
Lot V 5-7-7— 5 " " " 5.24 " " " 7.52 ".

administration of labeled Vit. B_{12} to dogs.

Material and methods. Co^{60} Vit. B_{12} was received in 2 lots with specific activities of 0.885 and 0.95 uc/ug, respectively. Prior to the experiment dogs were kept in isolation for at least one month, during which time they were wormed and immunized to canine hepatitis and distemper. All animals were young and healthy. Hematologic values were normal. Dogs were maintained under anesthesia by intermittent injections of sodium pentothal through catheter in femoral vein. The labeled vitamin was administered through this vessel. Under aseptic conditions the common bile duct was exposed and a polyethelene catheter inserted and tied at a point below the major bifurcation. The other end of catheter was connected to plastic bag for collection of bile under sterile conditions. Postoperatively, the dogs were fed a low fat diet including 0.3 g of Desicol. Two mg of Menadione were injected intramuscularly daily. At varying intervals the bag was emptied, volume of bile measured, and radioactivity/ml determined in a well-type scintillation counter. Counts/minute were converted to microcuries by reference to an aliquot of the radiovitamin injected.

Results. Table I shows dose of Co^{60} B_{12} injected, period of collection, volume of bile excreted, and total Co^{60} radioactivity measured. The experiment was terminated by irreversible plugging of the lumen or dislodgment of tubing from the common duct. Periods of collection ranged from 96 to 658 hours. Fig. 1 shows cumulative excretion of Co^{60} in bile of 5 dogs. Subjecting these excretion curves to graphic analysis a 2-component pattern was noted in dogs No. 379, 283 and 236. (illustrated in Fig. 2 for dog No. 379.) The half-times for the first (fast) components were 6.0, 6.5, and 7.5 hours, respectively. The slow components had half-times of 130, 90, and 295 hours, respectively. In dog No. 282 the collection period was not long enough to analyze the curve adequately, but 2 components were probable. The fifth



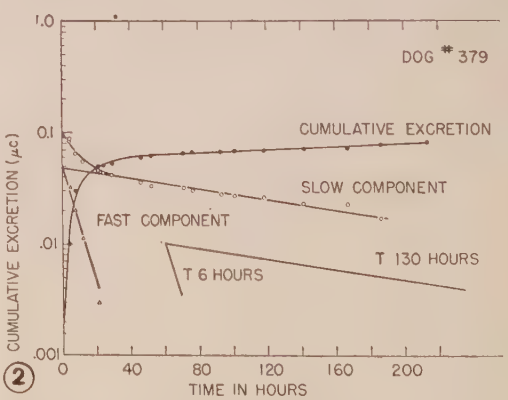


FIG. 1. Cumulative biliary excretion of Co^{60} in 5 dogs given intrav. cobalt labeled vit. B_{12}.

FIG. 2. Example of two-component biliary excretion of Co^{60} after intrav. inj. of cobalt labeled B_{12}.

TABLE II. Excretion of Co^{60} in Urine and Feces of Normal and Biliary Fistulae Dogs following Labeled B_{12} Injection.

	Dog No.	μc inj.	Days post-inj.	μc excreted in		Total μc	% excreted in		Total %
				Feces	Urine		Feces	Urine	
Normals	279	3.48	2		.05			1.4	
	281	3.48	5		.10			2.9	
	274	13.90	25	2.0	5.8	7.8	14.4	41.7	56.1
	237	4.85	54	1.9	.6	2.5	39.1	12.4	51.5
	275	8.12	235	2.9	1.4	4.3	35.7	17.2	52.9
	284	6.96	245	1.5	2.6	4.1	21.5	37.4	58.9
Biliary fistulae	282	3.48	5		.03			.9	
	283	3.48	7		.10			2.9	
	378	2.096	21 (12 days)*	.2	.05	.25	9.5	2.4	11.9
	379	2.096	21 (12 days)*	.2	.03	.23	9.5	1.5	11.0
	236	3.48	28	.55	.18	.73	15.8	5.2	21.0

* Fecal and urinary data available for 12 days only.

animal, No. 378, had a single component of excretion with a half-life of 145 hours (Fig. 2d).

Fecal excretion of radioactivity is shown in Table II for 5 dogs with biliary fistulae and 7 normal animals. It is clear that Co^{60} activity of bile does not explain total fecal excretion since dogs with biliary fistulae show radioactivity, though to a much lesser degree, in their feces.

Discussion. In general, presence of a 2-component excretion pattern indicates existence of 2 or more compartments in which the radioactive tracer is presumably diluted. Whether these compartments are in parallel, turning over the labeled vitamin independently and at different rates, or whether they are connected in series and are mutually dependent, cannot be ascertained in these experiments.

At present it is not possible to explain the apparent 2 component biliary excretion of radioactive cobalt after intravenous injection of the labeled vitamin. These observations in dogs are consistent, however, with those of Grasbeck *et al.*(4) in patients.

The physiologic significance of this 2 component excretion will have to be determined by further investigation. Certain facts are known which will help design further experiments. Meyer *et al.*(5) have shown that plasma binds radioactive B_{12} promptly and very tightly. In our preliminary experiments (unpublished) with continuous flow paper electrophoresis, the radioactivity appears with the $\alpha 1$, globulin of plasma, suggesting that this protein fraction carries Vit. B_{12} from sites of absorption to deposition in tissue. That the parenchyma of liver is relatively permeable to this complex and permits its passage into the biliary passages is unlikely because plasma clearance(6) takes place in human beings too rapidly to allow this interpretation. Plasma clearance in the dog, however, has not been measured yet. The fast biliary excretion may represent loss of radioactive vitamin not bound to tissue components while the slower excretion may represent loss of cells or cellular debris containing radiovitamin or its cobalt degradation products, or loss of degradation products alone. We did not determine whether biliary radioactivity was associated with cells, large molecules, or was freely dialyzable. Even more pertinent is the question whether the radioactive cobalt still represents Vit. B_{12} or some of its degradation products. In fact, interpretation of all long term studies with radioactive Vit. B_{12} is dependent upon whether the radioactivity is still associated with B_{12} or its metabolic products. If radioactivity represents undegraded B_{12} it still may not be representative of B_{12} behavior. To be representative, the injected radiovitamin must be uniformly distributed in the total body pool of B_{12} and one must determine whether the ratios of labeled to cold B_{12} are constant

throughout the body. We are not aware of any such determinations.

Since stools of dogs with biliary fistulae contain Co^{60} radioactivity, it is evident that labeled B_{12} or one of its degradation products passes into the lumen of the gastrointestinal tract from sources other than bile. Fecal radioactivity is obviously enhanced when the flow of bile to the intestines is uninterrupted. Whether Co^{60} in bile is still part of the Vit. B molecule and can be absorbed for reutilization has not yet been determined. Okuda *et al.* (7) have also independently demonstrated the presence of radioactive B_{12} in bile and stools of rats after injection of labeled B_{12} in rats and believe that fecal B_{12} has a biliary and an intestinal source.

Conclusions. 1. Following intravenous administration of 2.09 to 3.48 μc Co^{60} Vit. B_{12} to dogs between 2.6 and 6.6% of radioactivity was recovered in the bile. 2. In 3 of 5 dogs the 2-phased excretion pattern showed fast and slow components of 6.0 to 7.5 hours and 90 to 295 hours, respectively. 3. Fecal excretion of radioactivity in dogs with biliary fistulae was considerably less than in intact normal animals.

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Oxygen Consumption of the Hypothermic Potassium Arrested Heart.* (24255)

Robert M. Berne, Richard D. Jones and Frederick S. Cross

Division of Surgery, St. Luke's Hospital, and Department of Physiology, Western Reserve School of Medicine, Cleveland, O.

Cardiac arrest induced by intracoronary administration of potassium(1) or acetylcholine(2) has been found useful in cardiac surgery. However, revival of arrested hearts has not been completely satisfactory(3). Refractory ventricular fibrillation and inability of the previously asystolic heart to maintain an adequate cardiac output have been the principal difficulties. Previous studies(3) have indicated that the combination of cardiac hypothermia and potassium arrest resulted in the most rapid and complete post-arrest recoveries. The assumption was made that a reduction in myocardial oxygen requirement by cardiac hypothermia in the arrested heart was in large part responsible for these results and the present study was undertaken to test this asumption by quantitating the oxygen consumption of the normothermic and hypothermic arrested heart.

Methods. Fifteen technically satisfactory experiments were performed on open chest dogs anesthetized with sodium pentobarbital (30 mg/kg). The chest was opened bilaterally in the fourth intercostal space and the sternum transsected at this level. Following administration of heparin, the left coronary artery and the coronary sinus were cannulated. During control studies when the circulatory system was intact and the heart doing work, the source of blood for left coronary artery perfusion was either the dog's carotid artery or a pump oxygenator(4). When the pump oxygenator was used, blood was withdrawn from the right atrium at a rate

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equal to that of infusion into the left coronary artery. Blood from the coronary sinus was returned to the animal *via* the right jugular vein. Coronary blood flow was measured with an optically recording rotameter(5), and perfusion pressure with a Statham strain gauge. The oxygen content of coronary artery and coronary sinus blood was determined in duplicate by the method of VanSlyke and Neill (6). After coronary blood flow had been determined and blood samples for oxygen had been withdrawn in the normothermic working heart, the dog was placed on a complete cardiac bypass(4). The left coronary artery was supplied with oxygenated blood from the pump oxygenator at the same flow rate that existed during the control period. A catheter in the right atrium served to drain blood that returned by routes other than the coronary sinus. Potassium chloride was then infused into the tubing leading to the cannulated left coronary artery at a constant rate sufficient to produce and maintain left ventricular arrest. Since potassium has a coronary vasodilator or vasoconstrictor effect, depending upon dose employed(7,8), it was necessary to make careful adjustments of perfusion pressure and rate of potassium chloride infusion in order to keep coronary blood flow constant throughout the experiment. A small glass coil immersed in a water bath was interposed between the rotameter and the coronary artery cannula. Blood passing through the coil could be warmed or cooled by varying the temperature of the water bath. The temperature of coronary artery blood was measured at the coronary cannula and continuously recorded by a thermocouple connected to a Leeds and Northrop Speedomax. At the conclusion of each experiment the perfused portion of the heart was stained with India ink, excised, and weighed.

Results. The average coronary blood flow and oxygen consumption of intact working hearts were 73 and 9.18 ml/min/100 g of heart muscle, respectively (Table I). Cardiac arrest in the normothermic heart resulted in an average reduction in myocardial oxygen consumption to 2.08 ml/min/100 g of heart whereas cooling of the arrested heart to 21.5 to 25°C produced an average myocardial oxygen consumption of 0.66 ml/min/100 g of myocardium. In only one experiment (#8) was oxygen consumption of the cold arrested heart greater than that of the warm arrested heart. In this experiment, however, the myocardial oxygen consumption of the arrested heart was less than 0.2 ml/min at 36.5°C.

TABLE I. Oxygen Consumption of Normothermic and Hypothermic Potassium Arrested Hearts.

CBF	Myocardial oxygen consumption		
	Beating heart	K-arrested warm	K-arrested cold
	cc/min./100 g heart		
78	7.12 (34.0)*	.23 (34.0)*	<.20 (25.0)*
58		1.01 (37.0)	.64 (25.0)
83	10.61 (37.5)	1.08 (36.5)	.75 (23.0)
84		3.75 (36.5)	.39 (23.5)
67	9.51 (37.5)	2.41 (37.0)	1.27 (22.0)
117	8.42 (36.5)	3.74 (37.0)	.47 (23.0)
54	8.01 (36.5)	3.10 (36.5)	2.84 (22.5)
60	7.38 (36.5)	<.20 (36.5)	.48 (24.0)
72	11.01 (38.0)	2.00 (37.0)	<.20 (22.0)
51		1.53 (38.0)	<.20 (25.0)
50		2.65 (38.0)	.77 (22.0)
78	4.68 (36.5)	.94 (38.0)	<.20 (23.0)
† 124	14.87 (35.5)	2.17 (37.5)	.38 (22.0)
† 45		2.49 (36.0)	.65 (22.0)
† 83		4.05 (37.0)	1.11 (21.5)
Avg 73	9.18	2.08	.66

* No. in parentheses denotes temperature of blood perfusing left coronary artery in degrees centigrade.

† 2-4 separate determinations on the same heart.

Arterial oxygen content avg 20 vol % and in none of the experiments was it less than 17 vol %.

Discussion. Oxygen consumption of the normothermic potassium arrested heart in the present study was 2.1 ml/min/100 g of perfused heart. This is equivalent to 23% of oxygen consumption of the intact beating heart. These figures are in good agreement with those of McKeever *et al.*(9) who found oxygen consumption of the potassium arrested heart to be 2.2 ml/min/100 g of myocardium, which was between 16 and 32% of the oxygen consumption of the working ventricle in their studies. The values for oxygen consumption of the warm arrested heart in the present study are also in reasonable agreement with those of Berglund *et al.*(10). According to rough calculations from Fig. 2 of their paper, the average cardiac oxygen consumption for

6 experiments was 1.4 ml/min/100 g of heart, which was about 18% of the oxygen utilization of the working heart.

Cooling the blood perfusing the arrested heart reduced myocardial oxygen consumption to about one-third of that observed at normal body temperature and to 7% of the oxygen consumption of the intact working heart. These observations indicate that the rapid post-arrest recovery of the cold arrested heart is most likely due to its very low oxygen requirement. The possibility cannot be excluded, however, that the beneficial effects of cold in the arrested heart are not related to the reduction in myocardial oxygen consumption, but to some specific effect of low temperature on the metabolic characteristics of the myocardium. Application of these data to surgical situations in which cardiac arrest is necessary, suggests that coronary blood flow can be interrupted about 3 times longer in the hypothermic than in the normothermic arrested heart.

Summary. Oxygen consumption of the potassium arrested heart averaged 2.1 ml/min/100 g of perfused heart at normal body temperature, and 0.66 ml/min/100 g of perfused heart at 21.5 to 25°C.

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Significance of Circulating Phenols in Anemia of Renal Disease.* (24256)

I. DUNN, I. M. WEINSTEIN, M. H. MAXWELL AND C. R. KLEEMAN

Wadsworth Hosp., Veterans Admin. Center, and Dept. of Medicine, University of California Medical Center, Los Angeles

Anemia is a frequent complication in patients with chronic renal disease. The degree of anemia is roughly proportional to the level of azotemia, but the exact mechanism whereby anemia is produced is still unsolved. The most widely supported theory is that anemia results from a suppression of erythropoiesis by "toxic" metabolic products retained in the blood(1-5). Recent reports also stress the importance of increased erythrocyte destruction associated with inadequate bone marrow response(1-5). The hemolytic process is thought to be extracorpuscular, but no definite mechanism has been set forth. Evidence has appeared to suggest that phenol and/or its derivatives are hematopoietic poisons(6, 7). Conceivably they could be implicated either as bone marrow "toxins" or hemolytic agents. Most earlier work suffered from lack of specific analytic reactions employed to detect blood phenols(6). Using one such nonspecific reaction, the xanthoprotein index, Bock and Thedering(7) reported that elevated xanthoprotein levels were seen in anemic patients with renal insufficiency, and suggested that these substances might be implicated in production of anemia of these patients. Schmidt, *et al.*(8) applied a more specific technic for detection of blood phenols

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TABLE I. Levels of Ether Soluble Phenols in Blood of Patients with Renal Insufficiency.

Diagnosis	Avg packed cell vol, %	Avg hemoglobin, g %	Avg† creatinine, mg/%	Ether soluble phenols*			
				Free phenol-cresol	Conj. phenol-cresol	Free phenolic acids	Conj. phenolic acids
Chronic glomerulonephritis	26	8.3	11.3	*.34*	*.52*	*.70*	*.78*
Idem	39	13.8	3.5	.01	.06	*.15*	.15
"	35	12.5	3.2	.01	*.37*	*.16*	.09
Chronic pyelonephritis	32	11.2	*8.4	.02	.18	*.17*	*.33*
Idem	35	11.6	8.4	.01	*.86*	*.29*	*.35*
"	39	13.2	6.5	.02	*.52*	.11	.20
"	34	11.6	11.6	.01	*.82*	*.41*	*.98*
Chronic glomerulonephritis	33	10.0	10.1	.00	*1.12*	*.20*	*.54*
Polycystic kidney	48	14.9	6.5	.00	*.45*	*.19*	*.43*
Chronic glomerulonephritis	49	16.6	2.6	*.24*	.11	*.23*	.17
Chronic pyelonephritis	19	8.3	15.9	.03	*1.10*	*.45*	*1.73*
Idem	19	8.3	27.6	.03	*1.19*	*1.19*	*1.97*
Chronic glomerulonephritis	25	8.5	1.6	.07	.04	.02	.04
Idem	26	8.6	28.8	.01	*.73*	*.13*	*.31*
"	30	9.8	12.2	.03	*.87*	*.77*	*2.12*
"	28	10.0	13.2	.02	*.93*	*.58*	*1.86*
Chronic pyelonephritis	24	8.4	10.5	.01	.13	*.23*	*.54*
Acute glomerulonephritis	34	11.6	2.2	.01	.02	.06	.02
Chronic glomerulonephritis	35	13.0	5.4	.03	*.58*	*.20*	.13
Severe nephrosclerosis	35	11.3	6.5	.03	*.64*	*.80*	*.47*

* Free and conjugated phenols expressed as mg % phenol; free and conjugated phenolic acids expressed as mg % p-hydroxyphenylacetic acid. Range of normal values (5 subjects): Free phenol-cresols, .01-.04; conjugated phenol-cresols, .01-.19; free phenolic acids, .02-.11; conjugated phenolic acids, .03-.20. Italicized figures indicate elevated findings.

† Normal range: .8-1.5 mg %.

from patients with a variety of diseases. They found no elevation of free circulating phenol and cresol in 5 patients with renal disease. The present study investigates the role of these compounds in anemia of chronic renal disease.

Methods. Routine hemograms and phenol determinations were performed on normal subjects, and on patients with documented renal disease. Serum creatinine determinations were performed on all renal patients by a technic employing the usual Jaffe reaction (9). Medications that interfered with the phenol reaction, such as aspirin, were withheld for 24-48 hours prior to blood letting. *Blood phenol determination*: A modification of the method of Schmidt, *et al.*,(7), was used. Heparinized blood samples were drawn and the plasma frozen at –20°C until used. Four ether-soluble fractions were determined: free phenols (phenol and cresol), free aromatic hydroxy acids (*e.g.*, p-hydroxyphenylacetic acid, p-hydroxybenzoic acid) and the conjugates of each of these groups. Blood filtrates were prepared in 1:5 dilution with tungstic acid and separation of the phenolic constituents accomplished by continuous extraction with ether at pH 10 and 1. Conjugates were released as free compounds after preliminary extractions by hydrolysis with sulfuric acid. The alkalinized extracts were then evaporated to remove the ether and treated with Folin-Ciocalteu reagent. Readings were made on a Beckman Model B spectrophotometer at wave length of 740 mμ.

Results. The results of these studies are

illustrated in Table I. A significant elevation of *free* phenol and cresol was found in only 2 instances in 20 patients with renal disease and azotemia; in one of these, no anemia was present. Conjugated phenols tended to increase with degree of anemia and azotemia, but the relationship was not consistent. This same relationship held between severity of anemia and level of serum creatinine.

Discussion. It would appear that uremic patients are able to conjugate potentially toxic free phenols despite severe derangement of kidney function. The site of this "detoxifying" process is unknown, but presumably it is mainly in the liver. It is unlikely that these conjugated phenols can be implicated *per se* in pathogenesis of anemia of renal insufficiency. Previous investigations have shown that larger amounts of free phenolic acids and conjugated phenols than those detected in our patients do not produce any significant deleterious effect on the blood(6,8).

It is still possible that minute amounts of circulating free phenol and cresol might accelerate naturally occurring plasma lysins, and evoke a hemolytic reaction. Such a mechanism has been postulated by Ponder (10) to explain the hemolytic effect of naphthalene and other agents. However, it is difficult to accept this possibility in relation to free phenol and cresol in uremic patients inasmuch as the levels of these circulating compounds are not greater than in normal subjects.

Summary. Free and conjugated ether-soluble phenols in blood of uremic patients have been determined by a specific and reliable method. Free phenol and cresol are not elevated in the great majority of these patients. Reasons are given for discounting the role of elevated conjugated phenols and free phenolic acids in the pathogenesis of the anemia of chronic renal disease.

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Effect of a High Protein and High Nucleic Acid Diet on Occurrence of 2- Acetylaminofluorene-induced Cancer in Rats.* (24257)

W. F. DUNNING AND M. R. CURTIS

Cancer Research Laboratory, University of Miami, Coral Gables, Fla.

A previous report(1) showed an increased percentage of acetylaminofluorene-induced liver cancers in rats on a high protein diet. Fischer line 344 female rats on a synthetic diet containing 45% casein and 0.06% 2-acetylaminofluorene were compared with similar rats on a diet containing 26% casein. In the first group 11 of 12 rats died with liver cancers in an average of 303 days while only 6 out of 16 rats on the 26% casein diet developed liver cancers in an average of 283 days.

Harris(2) found no difference in incidence of induced liver cancers in Wistar rats on diets containing 0.04% acetylaminofluorene and a smaller difference in casein content, *i.e.* between 20 and 13% or with the addition of 3% liver extract. Morris, Wagner and Velat (3) reported a higher incidence of induced

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TABLE I. Composition of Diets.

Material	Diet 6	Diet 13	Diet 14	Diet 15	Diet 16	Diet 17
	%					
Casein	45.0	40.0	44.0	43.0	40.0	40.0
Brewers yeast	0	0	0	0	5.0	0
High nucleic acid yeast*	0	5.0	0	0	0	0
Desoxyribonucleic acid	0	0	1.0	2.0	0	5.0
Salt mixture	4.0	4.0	4.0	4.0	4.0	4.0
Cellu flour	2.0	2.0	2.0	2.0	2.0	2.0
Dextrin	34.0	34.0	34.0	34.0	34.0	34.0
Crisco	15.0	15.0	15.0	15.0	15.0	15.0
Halibut liver oil	.4	.4	.4	.4	.4	.4
2-Acetylaminofluorene	.06	.06	.06	.06	.06	.06

Vitamin supplement per kilo of diet†

	mg		mg		mg
Thiamin	4	Niacin	4	Choline HCL	2,000
Riboflavin	8	Calcium pantothenate	20	α-tocopherol	150
Pyridoxine HCL	4				

* Supplied through the courtesy of Dr. Joel B. Peterson, Standard Brands, Inc., New York City.

† The crystalline B vitamins were dissolved in distilled water and added as a daily supplement to each food cup.

mammary gland and ear-duct cancers and no difference in incidence of induced liver cancers in rats fed equal amounts of acetylaminofluorene with 10 μg compared with 1 or 2 μg of riboflavin added per gram to a semi-synthetic diet containing 24% casein.

In a preliminary experiment in our laboratory the average survival of rats on a 45% casein diet was increased from 317 days to 359 days by substitution of a high nucleic acid (6%) yeast for 5% casein. The added yeast significantly delayed the occurrence of the liver cancers but did not prevent their development. It seemed of interest to determine whether or not the protection resulted from the increased vitamin or nucleic acid content of the diet, or whether added protection might result from increased proportions of dietary nucleic acid.

In the current experiment 1, 2 and 5% desoxyribonucleic acid and 5% yeast were substituted for casein in a 45% casein diet.

Material and methods. Observations were completed on 6 groups of pedigreed Fischer line 344 female rats that received isocaloric portions of synthetic diets containing 0.06% 2-acetylaminofluorene. The diets varied only in proportion of casein, yeast or purified desoxyribonucleic acid as shown in Table I.

The rats were 4 to 5 months of age at start of the experiment. Each rat was housed in an individual cage with free access to water. The daily portion of 7 g of food was weighed out, and ½ cc of distilled water containing the daily supplement of crystalline B vitamins was added to each cup which was shaken briefly to mix in the vitamins before it was placed in the cage. Attempts were made to recover, weigh, and record any food that was spilled. Each gram of diet was equivalent to 4.7 calories, and the daily portion was 33 calories or a little less than the *ad libitum* consumption.

Each rat was weighed and inspected for tumors once a week. At death, post mortem examination included description of all visible tumors, gross sectioning of lungs and mammary glands, and inspection and weight of the liver, kidneys, adrenals, pituitary, and sex glands. Representative sections of each of these tissues and organs were preserved, and prepared sections were examined microscopically.

Results. The results are summarized briefly in Tables II, III, and IV. Table II shows that average daily consumption for the 6 groups of rats varied from 5.9 to 6.4 g of diet and the average total dose of 2-acetylaminofluorene ingested per rat varied from 1.1 to 1.6 g for the rats of the 6 groups. In each case consumption was less for the rats that received 1% nucleic acid and higher for the rats

TABLE II. Number of Rats in Each Group, Average Initial Body Weight, Daily Food Consumption, and Dose of 2-Acetylaminofluorene.

%	No. rats	Body wt (g)	Avg daily ration (g)	Avg total dose AAF (g)	Avg daily dose AAF (mg)
45 casein	16	145	6.3	1.3	3.8
5 nucleic acid yeast	18	147	6.4	1.6	3.8
1 nucleic acid	12	138	5.9	1.1	3.5
2 " "	12	146	6.2	1.2	3.7
5 " "	11	136	6.2	1.2	3.7
5 Brewer's yeast	12	138	6.3	1.2	3.8

that received the supplement of high nucleic acid yeast. Average daily consumption of acetylaminofluorene, however, varied only from 3.5 to 3.8 mg per rat per day.

From Table III it appears that average terminal body weight was considerably less than weight at the start of the experiment for rats of each group. An average weight loss of 14% was observed for the rats that received 5% Brewers yeast and more than twice that or 32% was observed for the rats on the diet containing 1% nucleic acid. The livers were from 2½ to 3 times larger than normal average liver weight and average kidney weights were slightly higher than normal for all groups. Otherwise there were no significant changes in the organs that were weighed.

Table IV shows average survival period for the rats of each group and the percentage of induced neoplasms that were observed. The rats with the 5% substitution of high nucleic acid yeast survived an average of 409 ± 9.4 days or significantly longer than the average survival (349 ± 11. days) for the group on the 45% casein diet or the average of 317 ± 6.5 days for the rats with 5% Brewers Yeast substitution. There were no significant differences between the groups that received different quantities of nucleic acid.

Malignant hepatoma were observed in from 67 to 92% of the rats of the 6 groups. There appeared to be no noteworthy differences in morphology or degree of malignancy except that lung metastases were observed in only 30% of the rats of the group that received 5% nucleic acid compared with 67% for the rats on the control or 45% casein diet.

Squamous cell carcinoma of the external auditory meatus were observed in 33 to 75% of the rats of the 6 groups. This type of neoplasm has been observed previously in about 20% of the Fischer line 344 rats on various dietary regimens containing 26% protein. Possibly the high protein diet accelerated occurrence of these tumors because the observed frequency was higher than expected in all 6 groups. In the rats with added nucleic acid the average latent period was only 280 to 290 days. The neoplasms were bilateral in 6 rats, 6 showed metastases to the lungs and one dissemination to the pituitary gland.

Adenocarcinomas of the mammary gland were observed in 4 or 25% of the rats on the control or 45% casein diet and in only one of the rats on the high nucleic acid yeast diet and in none of the rats of the other groups. In addition 3 rats, one each from the control, and 1, and 2% nucleic acid diets had squamous cell carcinoma of the bladder. These neoplasms had been observed previously only on

TABLE III. Number of Rats on Each Diet, Average Initial and Final Body Weight, and Percentage Weights of Organs.

		Wt (g)		Organ wt in % of body wt					
Group	No. rats	Initial	Final	Liver	Kidney	Adrenal	Pituitary	Ovary	Uterus
Diet 6	16	145	117	15.8	1.5	.05	.01	.05	.39
13	18	147	112	15.8	1.4	.04	.01	.04	.42
14	12	138	93	14.7	1.5	.05	.01	.05	.36
15	12	146	102	15.7	1.5	.05	.01	.04	.45
16	11	136	116	15.7	1.3	.05	.01	.05	.37
17	12	138	107	12.8	1.4	.05	.01	.04	.40

TABLE IV. Average Survival in Days ± P.E. and % of Rats with Induced Cancer.

Group %		No. rats	Survival, days ± P.E.	Liver cancer %	Liver cancer % met.	Ear cancer %	Ear cancer Avg days	Mammary cancer %	Mammary cancer Avg days
45	casein	16	349 ±11.	75	67	44	323	25	327
5	high nucleic acid yeast	18	409 ± 9.4	83	47	33	338	6	406
1	nucleic acid	12	324 ±11.	67	62	67	290	0	
2	" "	12	322 ± 6.1	92	54	50	280	0	
5	" "	12	315 ± 6.1	83	30	75	286	0	
5	Brewer's yeast	11	317 ± 6.5	82	62	64	319	0	

diets containing added tryptophan(1) or indole(4). An adenocarcinoma of the lungs that was probably primary was observed in one rat on the 2% nucleic acid diet. Two rats that received the Brewers yeast supplement had mixed tumors (adenocarcinoma and osteosarcoma) of the illium. One rat on the high nucleic acid diet had a fibrosarcoma of the right fore leg with lung and lymph node metastases and another had an adenoma of the adrenal.

Summary. 1. The substitution of 5% high (6%) nucleic acid yeast for casein in a synthetic diet containing 45% casein and 0.06% 2-acetylaminofluorene significantly increased average survival of the rats but did not prevent development of the induced neoplasms. 2. Substitution of 1, 2, or 5% purified desoxyribonucleic acid or 5% Brewers yeast for casein in similar diets did not affect average survival period or frequency of occurrence of the induced neoplasms. 3. The high protein diet appeared to accelerate occurrence of the acetylaminofluorene-induced squamous cell carcinomas of the external auditory meatus.

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The Role of Indole in Incidence of 2-Acetylaminofluorene-Induced Bladder Cancer in Rats.* (24258)

W. F. DUNNING AND M. R. CURTIS
Cancer Research Laboratory, University of Miami, Coral Gables, Fla.

Previous reports(1,3) have shown that addition of 1.4% DL-tryptophan to a synthetic diet containing 25% tryptophan-free casein hydrolysate and 0.06% 2-acetylaminofluorene increased from 37 to 75 the percentage of rats that developed liver cancer and appeared to be an etiological factor in the incidence of bladder cancer. A study by Glass and Plaine (4) of the enhancing effect of dietary tryptophan on appearance of melanotic tumors in the fruit fly *Drosophilia melanogaster* showed that indole was the effective promoting agent.

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In rats indole-acetic acid is the natural metabolite of tryptophan and indole results from action of intestinal bacteria. It seemed therefore, of interest to determine whether or not either indole or indole acetic acid were responsible for the observed enhancing effect of dietary tryptophan on the incidence of bladder cancer in these rats.

Material and methods. Pedigreed Fischer line 344 female rats 4 or 5 months of age were divided into 4 groups and placed on the synthetic diets to be described. Each rat was housed in an individual cage with free access to water. A daily portion of 7 g of diet or ap-

TABLE I. Composition of Diets.

Material	Diet 30	Diet 31	Diet 32	Diet 33
	%			
Casein	25.0	25.6	24.8	25.4
Salt mixture	4.0	4.0	4.0	4.0
Cellu flour	2.0	2.0	2.0	2.0
Dextrin	52.0	52.0	52.0	52.0
Crisco	15.0	15.0	15.0	15.0
Halibut liver oil	.4	.4	.4	.4
DL-tryptophan	1.4	0	0	0
Indole	0	.8	1.6	0
Indole acetic acid	0	0	0	1.0
2-acetylaminofluorene	.06	.06	.06	.06

proximately 33 calories was weighed out and presented in a food cup to which was added the daily supplement of crystalline vitamins.

The composition of the diets is shown in Table I. Each diet contained approximately 26% protein (casein), 52% carbohydrate (dextrin), 15% fat (crisco) plus adequate minerals and vitamins. The essential variants were 1.4% DL-tryptophan for diet 30; 0.8% indole for diet 31; 1.6% indole for diet 32; and 1.0% indole acetic acid for diet 33. Diet 30 served as the control diet for this experiment and was similar in composition to the previously reported(3) experimental Diet 4 except that the 1.4% DL-tryptophan was added to intact casein instead of the tryptophane-free casein hydrolysate. All of the diets contained the same quantity or 0.06% 2-acetylaminofluorene and the following vitamin supplement per kilo of diet:

	mg
Thiamin	4
Riboflavin	8
Pyridoxine HCL	4
Niacin	4
Calcium pantothenate	20
Choline HCL	2,000
α-tocopherol	150

Each rat was weighed and inspected for tumors once a week. At death, post-mortem examination included description of all visible tumors, gross sectioning of lungs, bladder and mammary glands and inspection and weight of the liver, kidneys, adrenals, pituitary, thyroid and sex glands. Representative sections of each of these tissues and organs were preserved and examined microscopically.

Results. Table II shows the number of rats in each group, average initial body weights, daily food consumption, and daily and total dose of 2-acetylaminofluorene ingested. The 4 groups varied very little in either calorie or drug intake. Average daily caloric intake varied from 28 to 29 calories and average daily dose of 2-acetylaminofluorene ingested varied from 3.5 to 3.7 mg.

Table III shows average post-mortem body weight and percentage weights of some of the organs from the rats of each group. The rats that received 1.0% indole acetic acid and 1.6% indole lost more than 20% in total body weight. The group that received added DL-tryptophan lost an average of 15% in total body weight. Rats of the group that received 0.8% indole sustained an average loss of 8%. The livers were proportionately larger in the rats that received DL-tryptophan and indole acetic acid than they were in the other groups and the thyroids were larger in the 2 groups that received indole. Otherwise there appeared to be no consistent differences in the organs that were weighed.

Average survival period and percentage of induced neoplasms is shown in Table IV. A group of rats that received a synthetic diet deficient (0.14%) in tryptophan from a previous experiment(2) has been included for comparison with the currently reported groups. Average survival period varied from 334 days for rats on the diet containing 1.0% indole acetic acid to 436 days for the rats that

TABLE II. Number of Rats in Each Group, Average Initial Body Weight, Daily Food Consumption, and Dose of 2-Acetylaminofluorene.

Group %	No. of rats	Body wt (g)	Daily ration (g)	Daily ration (cal.)	Total dose AAF (g)	Daily dose AAF (mg)
1.4 DL-tryptophan	12	124	5.9	28	1.3	3.5
1.0 Indole acetic acid	12	124	6.1	29	1.2	3.7
.8 Indole	12	126	6.0	28	1.5	3.6
1.6 "	12	131	6.2	29	1.6	3.7

TABLE III. Average Post Mortem Body Weight, and Percentage Weights of Some Organs.

%	Group	No. of rats	Body wt (g)	Organ wt in % of body wt						
				Liver	Kidney	Adrenal	Pituitary	Ovary	Uterus	Thyroid
1.4	DL-tryptophan	12	105	17.6	1.2	.04	.01	.05	.34	.01
1.0	Indole acetic acid	12	95	18.1	1.4	.04	.01	.05	.34	.01
.8	Indole	12	116	14.0	1.2	.03	.01	.04	.34	.02
1.6	"	12	97	10.3	1.2	.05	.01	.05	.36	.02

TABLE IV. Average Survival in Days, and Percentage of Rats with Induced Cancers.

%	Group	No. of rats	Days to death	% with cancer of			
				Liver	Ear	Breast	Bladder
1.4	DL-tryptophan	12	360	91	25	17	75
1.0	Indole acetic acid	12	334	83	25	0	58
.8	Indole	12	407	83	25	17	83
1.6	"	12	436	83	0	0	83
.14	DL-tryptophan	11	352	55	0	9	0

received 1.6% indole.

Liver cancers were observed in all groups. All but one of the rats on Diet 30 with the 1.4% tryptophan added to intact casein and all but 2 rats in each group that received indole and indole acetic acid developed liver cancers. This may be compared with 8 out of 11 or 73% that developed liver cancers in the previously reported group that received 1.4% added DL-tryptophan. As previously mentioned the livers were larger in the rats that received 1.4% tryptophan and 1.0% indole acetic acid than in the rats that received indole. The tumors also were notably larger in the rats of these 2 groups.

Table V shows that malignant hepatoma (Fig. 1) were identified in all 11 of the rats that received 1.4% tryptophan and in 10 of the rats that received 1.0% indole acetic acid. Lung metastases (Fig. 2) composed of neoplastic primary liver cells were identified in 63 and 90%, respectively, of the rats of these 2 groups. In addition, 4 of the former and 6 of the latter had areas of cholangioma and adenocarcinoma. No metastases resembling these neoplasms were identified. Of the rats that received 0.8% and 1.6% indole 7 and 6, respectively, had neoplasms identified as malignant hepatoma with lung metastases identified in 4 and 2, respectively. In addition, 8 rats in each of the indole-fed groups had liver tumors that were classified as cholangioma or adenocarcinoma (Fig. 3) probably of bile duct origin. Metastases (Fig. 4) from these tumors were identified in the lungs of 3 rats that received 0.8% indole and in one rat that received 1.6% indole. In general the liver tumors in the indole-fed rats were smaller and more discreet than in the rats of the other groups so that the apparent difference in morphology may have resulted from errors in sampling. Only representative sections from each liver and lung were examined microscopically and in the larger livers and tumors of the rats that received 1.4% tryptophan and 1.0% indole acetic acid the tumors of bile duct origin may have been missed.

In previous studies tumors of the external auditory meatus have been observed in about 20% of Fischer line 344 female rats that have

TABLE V. Classification of Induced Liver Neoplasms, and Percentage with Metastases.

%	Group	Hepatoma	% with met.	Cholangioma and adenocarcinoma	% with met.
1.4	DL-tryptophan	11	63	4	0
1.0	Indole acetic acid	10	90	6	0
.8	Indole	7	57	8	38
1.6	"	6	33	8	12

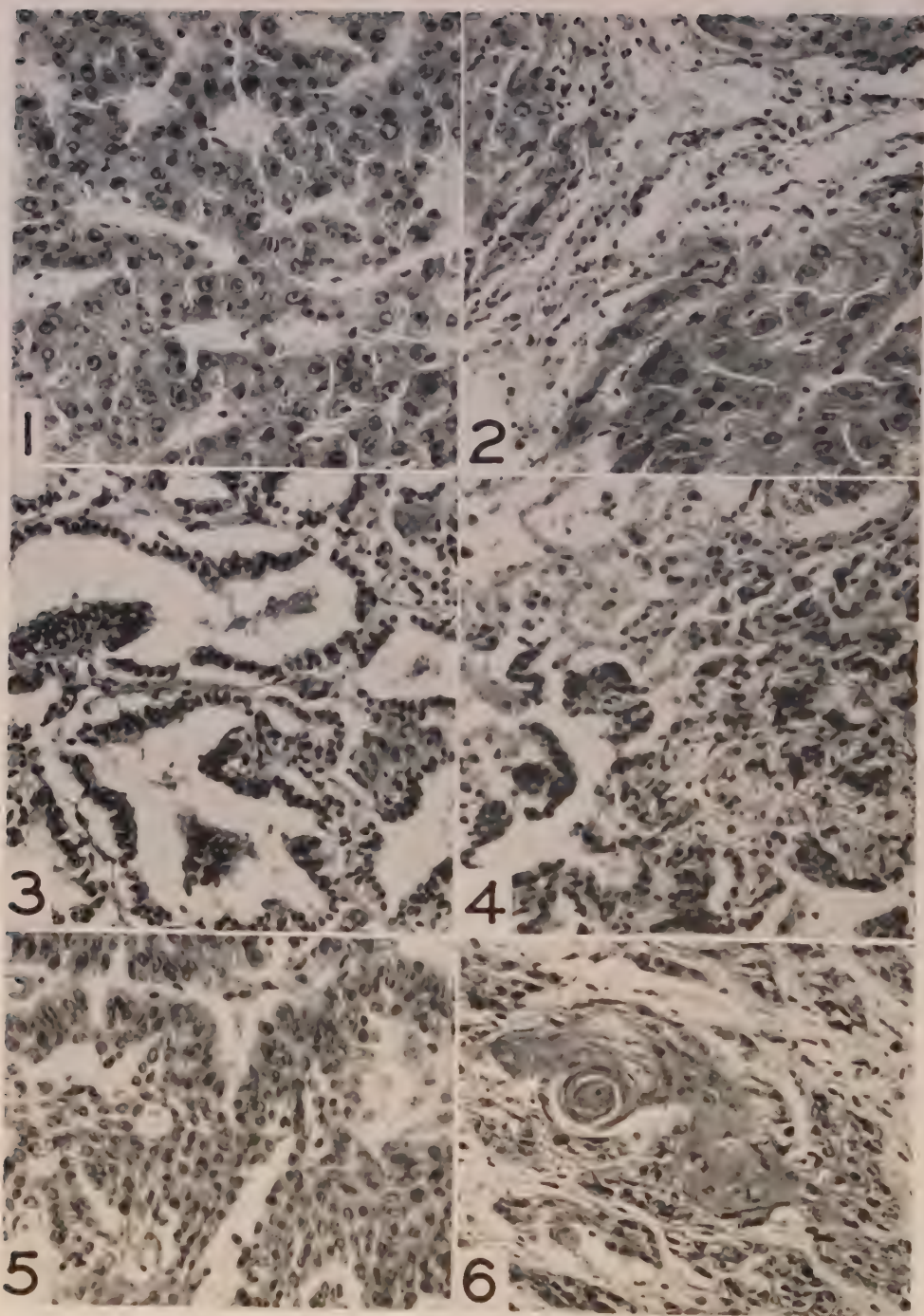

FIG. 1. Malignant hepatoma from rat 333 days after being placed on diet containing 1.0% indole acetic acid and 0.06% acetylaminofluorene. × 272.

FIG. 2. Multiple metastases in lungs of rat with malignant hepatoma shown in Fig. 1. × 272.

FIG. 3. Adenocarcinoma of liver in rat 553 days after being placed on diet containing 0.8% indole and 0.06% acetylaminofluorene. × 272.

FIG. 4. Metastases in lungs of rat with adenocarcinoma of liver shown in Fig. 3. × 272.

FIG. 5. Papillary carcinoma of bladder in rat 384 days after being placed on diet containing 0.8% indole and 0.06% acetylaminofluorene. × 272.

FIG. 6. Squamous cell carcinoma of the bladder from rat 477 days after being placed on diet containing 1.6% indole and 0.06% acetylaminofluorene. × 272.

been fed 0.6% 2-acetylaminofluorene. In this study 3 rats or 25% in each of 3 groups had tumors of the external ear at death. All 9 were squamous cell carcinoma.

Adenocarcinomas of the mammary gland were found in 2 rats that received 1.4% tryptophan and in 2 rats that received 0.8% indole.

Cancers of the urinary bladder were present in rats of all 4 groups. They were observed in 75% of the rats that received 1.4% DL-tryptophan; 58% of the rats that received 1.0% indole acetic acid; and in 83% of the rats of the 2 groups that received indole.

Previously bladder cancers were observed in 100 and 92%, respectively, of the rats that received 1.4 and 4.3% DL-tryptophan added to a tryptophane-free casein hydrolysate but were not found in rats on a 26% casein diet or in rats on a synthetic diet deficient in tryptophan that survived as long or longer than the rats that received added tryptophan or indole. The induced cancers were classified as papillary (Fig. 5) or squamous cell carcinoma (Fig. 6) or a combination of both types. In many instances the cancerous growth filled and distended the lumen of the bladder. From these results it is apparent that indole is as effective as tryptophan in the initiation of these neoplasms.

Summary. 1. Fischer line 344 female rats were fed 2-acetylaminofluorene in synthetic diets containing 25% casein and supplements of 1.4% DL-tryptophan; 1.0% indole acetic acid; 0.8% indole and 1.6% indole. Liver cancers developed in 91% of the rats of the first group and in 83% of the rats of the other 3 groups. 2. The majority of the liver neoplasms in the rats that received 1.4% DL-tryptophan and 1.0% indole acetic acid were malignant hepatoma and lung metastases were present in 63% and 90% respectively. 3. The majority of the liver neoplasms in the rats that received 0.8% and 1.6% indole were cholangioma or adenocarcinoma and lung metastases from these tumors were identified in 38 and 12%, respectively. 4. Cancers of the urinary bladder were observed in 75% of the rats that received 1.4% DL-tryptophan; 58% of the rats that received indole acetic acid and in 83% of the rats that received indole. Indole appeared to be as effective as dietary tryptophane in the initiation of the bladder neoplasms. 5. The cancers of the urinary bladder were papillary or squamous cell carcinoma.

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Effect of Age at Ovariectomy on Mammary Gland Development in C3H/He Crgl Mice.* (24259)

RICHARD S. SMITH AND HOWARD A. BERN

Department of Zoology, Cancer Research Genetics Laboratory, and Department of Anatomy, School of Medicine, University of California, Berkeley

It is well recognized that in certain strains of susceptible mice, including the C3H strain (1,2), mammary tumors develop despite ovariectomy. The hormonal stimulus for genesis of these tumors has been ascribed to the adrenal cortex which, in several strains, undergoes a tumorous change leading to so-called nodular hyperplasia or to carcinoma(1-6).

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TABLE I. Degree of Alveolar Development of Mammary Glands of Virgin C3H/He Crgl Mice Ovariectomized at Various Ages.

Age at ovariectomy (mo)	No. of mice	Mean age at death & range (mo)	No. and (%) of mice showing various degrees of mammary alveolar development*						No. of mammary nodules per mouse†
			3	2	1	±	N	0	
Controls	53	15 (8–26)	12(23)	26 (49)	15 (28) (1 no T)‡	—	—	—	32.1 ± 3.9
2	8	23 (23)	—	2 (26) (1 no T)	3 (37) (1 no T)	3 (37) (3 no T)	—	—	6.4 ± 1.8
3	15	17 (13–23)	—	2 (13)	3 (20)	4 (27)	4 (27)	2 (13) (2 no T)	14.9 ± 4.7
4	19	15 (9–26)	—	—	—	2 (10)	14 (74) (1 no T)	3 (16) (1 no T)	11.5 ± 2.9
5	21	13 (8–30)	—	1 (5)	—	2 (9) (2 no T)	16 (77) (3 no T)	2 (9)	10.3 ± 2.3
6.5	29	16 (12–27)	—	—	—	—	24 (83) (2 no T)	5 (17) (2 no T)	11.2 ± 2.1
8.5	11	17 (11–21)	—	—	—	—	10 (91) (1 no T)	1 (9) (1 no T)	14.7 ± 3.5

* Rating scheme for mammary gland alveolar development: 0 = completely devoid of alveoli and nodules; N = devoid of normal alveoli, but hyperplastic nodules present on otherwise bare ducts (as in Fig. 3 and 4); ± = occasional alveolar buds and isolated alveoli; 1 = isolated alveoli and small clusters; 2 = many alveoli and small clusters (as in Fig. 2); 3 = definite lobuloalveolar development (as in Fig. 1).

† Mean ± stand. error of mean.

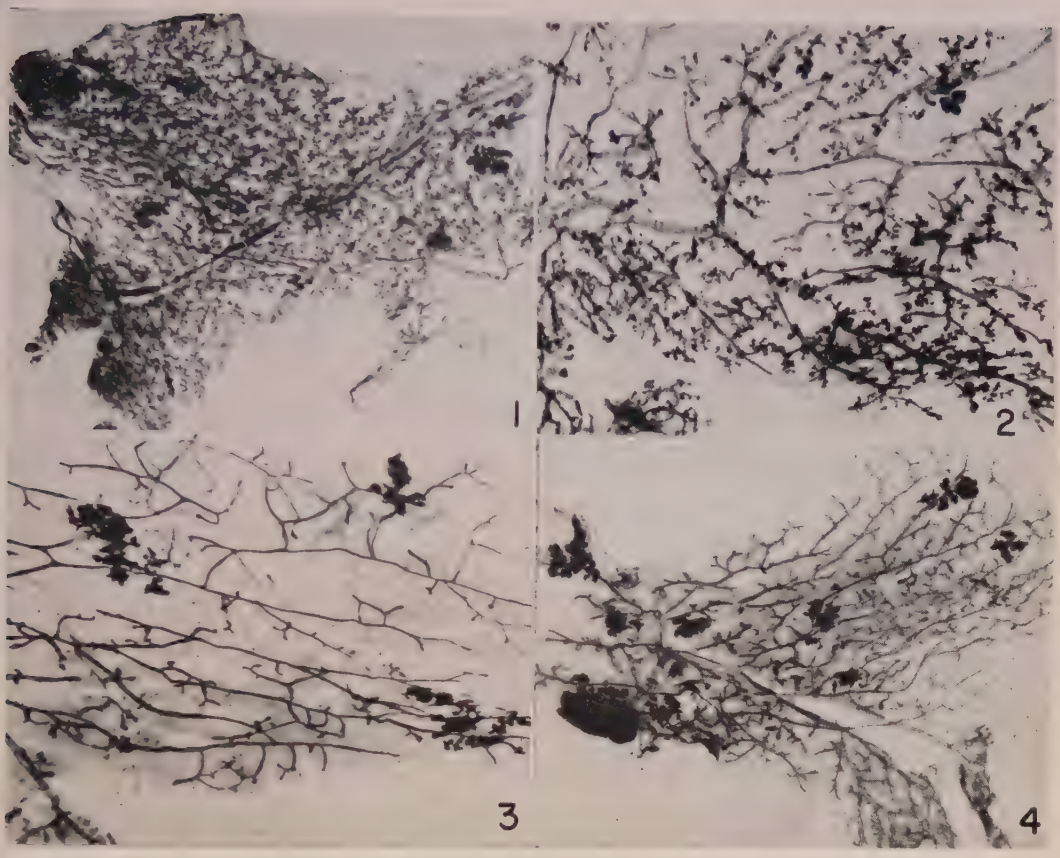
‡ "no T" = No. of mice in group without gross or microscopic tumors.

The mammary glands of mice ovariectomized before 2 months of age have been examined in certain strains. The C3H(2) and NH(4) strains showed less ductal and alveolar development than the non-ovariectomized controls; however, hyperplastic nodules were present in all groups. In the ce strain (3) the majority of the animals exhibited ductal development as the only form of mammary gland development; however, about 25% of the animals over 12 months old showed some alveolar development. In this strain no hyperplastic nodules were observed.

The present investigation was undertaken to determine whether ovariectomy performed at different ages would produce differences in state of stimulation of the mammary gland of our C3H/He strain at the time of mammary tumor development.

Materials and methods. A total of 148 virgin female mice of the C3H/He Crgl strain was used in this experiment. The experimental groups consisted of 15 animals bilaterally ovariectomized at 3 months of age, 19 at 4 months, 21 at 5 months, 29 at 6.5 months, and 11 at 8.5 months. Fifty-three animals were maintained as unoperated virgin controls. In most cases the animals were maintained until palpable mammary tumors appeared, at which time they were sacrificed. Twelve percent of the experimental animals died before showing tumors, and an additional 4% failed to develop tumors and were sacrificed between 23 and 30 months of age (nontumorous mice are indicated by "no T" in Table I). In addition, 8 animals serving as part of another experiment are included in Table I. This group was bilaterally ovariectomized at 2 months of age and sacrificed at 23 months of age.

At autopsy, the mammary glands were prepared as whole-mounts and stained with hematoxylin. The number of hyperplastic nodules and presence of mammary tumors were recorded. The state of alveolar development of the mammary gland was evaluated according to the rating scheme indicated in Table I. It is to be emphasized that judgment of degree of alveolar development was made without regard to extent of elaboration of the duct system and hence without regard to total mass of the gland.



All photographs are of whole-mounts of a No. 3 mammary gland stained with hematoxylin. × 5. All mice possessed palpable tumors.

FIG. 1. Control showing considerable lobuloalveolar development and several hyperplastic nodules (13 mo old at death).

FIG. 2. Ovariectomized at 2 mo showing many alveoli and small clusters as well as hyperplastic nodules (23 mo old at death).

FIG. 3. Ovariectomized at 5 months showing hyperplastic nodules on otherwise bare ducts as the only form of alveolar development (16 mo old at death).

FIG. 4. Ovariectomized at 6.5 months showing hyperplastic nodules, bare ducts, and a small tumor (15 mo old at death).

Results. Nodular hyperplasia of the adrenal cortex was evident in all experimental animals. At tumor age, the mammary gland ducts of the control animals were well developed and possessed many thin branches, in addition to showing active duct growth as indicated by terminal buds and "club ends." There was appreciable alveolar development in the control group varying from glands showing diffuse alveolar development with few lobules of alveoli to glands showing definite lobuloalveolar development (Fig. 1). Hyperplastic nodules were present in the mammary glands of all of the control animals, averaging 32 per animal, approximately 3 times the average number (12) seen in all the experimental groups.

The mammary ducts of the experimental groups showed considerably less branching than the controls (Fig. 1 and 2). Degree of branching was usually more extensive in animals castrated at later ages than those castrated at an early age (Figs. 2 and 4). In the groups castrated at 2, 3, and 4 months of age, wide ducts with short, thick branches predominated. Evidence of active duct growth was observed in some members of all experimental groups.

The mammary glands of animals ovariectomized at 2 months of age had fairly extensive alveolar development ranging from localized development of alveolar buds to large numbers of alveoli and many lobules. The average picture was that of alveoli throughout the glands with some lobule formation (Fig. 2). Uteri from these mice were large and showed cystic glands.

Ovariectomy at 3 months of age resulted in mammary glands with a wide range of alveolar development, *i.e.*, no alveolar development to large numbers of alveoli with some lobule formation. In 27% of the animals the only form of alveolar development was that of hyperplastic nodules on otherwise bare ducts.

Ovariectomy at 4 months resulted in a larger percentage (74%) of animals possessing hyperplastic nodules as the only form of alveolar development, with a small number showing either slight or no alveolar development.

Ovariectomy at 5 months resulted in approximately the same picture (Fig. 3) as that of the 4-month group, but included one animal with fairly extensive alveolar development. This exceptional animal possessed very large adrenals (totaling 24 mg) and a cystic uterus.

83% (24 mice) of the mice ovariectomized at 6.5 months showed glands containing hyperplastic nodules on otherwise bare ducts as the only representation of alveolar development (Fig. 4). Seventeen percent (5 mice) of this group possessed glands with neither alveoli nor nodules, although 3 of these mice had palpable tumors. Ovariectomy at 8.5 months resulted in essentially the same pattern as that of the 6.5-month castrate group. Uterine development in these two older groups was similar to that seen in the non-operated controls.

Within a single experimental or control group, there was no correlation between age at death (generally the same as tumor age) of the individual animal and degree of mammary gland development.

Discussion. Nandi(7) has determined for the C3H/He Crgl mouse that the minimum hormonal combination necessary for lobuloalveolar development of the mammary gland is estrogen, progesterone, and somatotropin *or* mammotropin. Relating these findings to the present investigation, it would appear that the nodular hyperplastic adrenal, at least in mice ovariectomized at 2 and 3 months of age, elaborates estrogen-like and progesterone-like substances in sufficient quantities to account for the observed lobuloalveolar development. In the groups ovariectomized at late ages (6.5 and 8.5 months), the nodular hyperplastic adrenal cortex contributes to a hormonal environment that is sufficient for induction and/or maintenance of hyperplastic alveolar nodules of the mammary gland but not of normal alveolar development. Thus, the steroid hormonal requirements for nodule formation, as opposed to generalized alveolar development, can be defined in terms of the secretions of the transformed adrenal cortex of a mouse ovariectomized late in life. This latter adrenal appears to be less efficient in replacing the ovary than is the adrenal from a mouse ovariectomized at an earlier age, judged both by mammary development and by uterine size.

Summary. C3H/He Crgl virgin female mice were ovariectomized at approximately monthly intervals between 2 and 8.5 months, and the mammary glands were examined at the time of mammary tumor development. Normal alveolar development of the mammary gland was greatest in animals ovariectomized at an early age and less in animals ovariectomized at later ages. In mice ovariectomized at 6.5 and 8.5 months, normal alveolar development was absent, the sole alveolar development being in the form of hyperplastic nodules. The secretory products of the transformed adrenal in the older mice thus appear to be adequate for development of hyperplastic nodules, but not for normal alveoli.

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Differences in Inositol Requirements of Several Strains of HeLa, Conjunctival and Amnion Cells.* (24260)

R. SHIHMAN CHANG†
Department of Microbiology, Harvard School of Public Health, Boston, Mass.

A minimal growth medium consisting of 27 factors capable of supporting the propagation of several strains of human cells for at least several months had been described by Eagle (1). Eagle and his associates(2) also reported that meso-inositol was an additional essential growth factor and that the requirement of the HeLa cell for inositol was irregular and was dependent on method of dialysis. Geyer and Chang(3) found that the HeLa and the conjunctival cells would show advanced degenerative changes after 4-7 days in Eagle's minimal growth medium, that addition of inositol prevented the appearance of cell degeneration, and that the HeLa cell regularly required inositol. Since the minimal growth medium described by Eagle is an important advance in the methodology of human cell culture, these conflicting reports prompted us to explore the basis of this irregularity. Among the factors studied are differences between strains of a cell, difference between stable laboratory cell lines and primary cell explants, contamination of cell culture by the pleuropneumonia-like organisms, and spontaneous appearance of nutritional variants and the extent of dialysis of serum. The results of these studies form the basis of this report.

Materials and methods. The methods were those described earlier(4). The minimal growth medium for determination of inositol requirement of the various strains of human cells consisted of 10% dialyzed human or horse serum in Eagles basal medium(1) plus inositol at concentration of 10 to 20 μM. The withdrawal of inositol resulted in advanced degeneration of the inositol-requiring strains within 5 to 10 days. Total inositol content of serum was analyzed, using *Saccharomyces cerevisiae* as assay organism(5).

Results. Differences in HeLa strains from several laboratories. The inositol requirement of several strains of HeLa cells, kindly furnished by other investigators, was determined. The strain maintained in the author's laboratory since 1954 invariably degenerated in the minimal growth medium without inositol. The strain obtained from Dr. B. Roizman, Johns Hopkins University, was found to propagate at approximately the same rate in the minimal growth medium with or without inositol for 14 days (Table I). Several other strains were found contaminated with a pleuropneumonia-like organism and were observed only for 7 days in the presence of the contaminant; 4 degenerated completely in medium without inositol while one gave inconclusive results.

Difference in conjunctival strains maintained in different growth media. The stock culture has been found on numerous occasions to degenerate in the assay medium without inositol(3). A subculture maintained in a 0.1 mM glucose medium (10% dialyzed serum in Eagle basal medium with the concentration of glucose reduced to 0.1 mM and with addition of inositol to a cencentration of 10-20 μM) for about 6 months failed to show similar changes. It showed a definite though small net increase in cell number during the first 2 weeks in the assay medium without inositol, and has since been propagating for at least 2 months (Table I).

Difference between a stable strain and fresh explants of amnion cells. The FL strain of

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† Senior Research Fellow, U. S. P. H. S.

TABLE I. Multiplication or Degeneration of Several Strains of HeLa, Conjunctival and Amnion Cells in Minimal Growth Media with and without Inositol.

Cell strain	Inositol in media	No. of cells on days No.* 0	7	14	28
HeLa (Chang)	0	18	0	0	
	10 μM	18	60	213	
HeLa (Roizman)	0	20	70	246	
	10 μM	20	105	296	
Conjunctival (stock)	0	23	0		
	10 μM	23	430		
Conjunctival (.1 mM glucose)	0	16	15	33	
	10 μM	16	41	113	
Amnion (FL)	0	17	0		
	10 μM	17	258		
Amnion (fresh explants)	0	136	49	17	15
	10 μM	136	66	22	9
	Whole serum†	136	134		334

* Avg No. of cells in thousands/culture tube.
† Same as assay medium except undialyzed serum was used.

amnion cell, isolated by Fogh(6) degenerated completely in the assay media without inositol. With cell suspensions freshly prepared from 3 human placentae by the trypsin method, no apparent difference could be observed in the assay media with or without inositol. It should be pointed out, however, the fresh amnion cell explants failed to show any net increase in number of cells over the observation period of 4 weeks in the assay media. The minimal growth medium is apparently less adequate in supporting growth of fresh amnion cell explant as compared to a medium containing undialyzed serum (Table I).

The results presented in Table I are those of representative quantitative experiments. Variations in the results of different experiments did exist; they were the date of onset of degeneration, date of apparent completion of degeneration and actual net increase in cell number. However, the pattern of advanced degeneration of inositol-requiring strains and varying degree of multiplication of inositol-independent strains when tested in the inositol-free medium has been consistently observed in every experiment. Clone lines from the Roizman and Chang strains of HeLa as well as the stock and .1 mM glucose conjunctival cells also gave similar results.

Isolation of inositol-independent variant through deliberate selection. The relative frequency of inositol-independent cells present in the inositol-requiring culture was explored using a method similar to that used successfully for isolation of carbohydrate variants(7). At each attempt, 3 to 5 million cells from the inositol-requiring culture were exposed to the minimal growth medium without inositol; the inositol-free medium was replenished on the 3rd, 5th and 7th days, then at weekly intervals until the appearance of inositol-independent variant or the end of 2 months if no variant developed. Advanced degenerative changes regularly appeared on about the 7th day which proceeded to completion except in one instance. Seven attempts have been made with the inositol-requiring HeLa strain; no inositol-independent variant has yet been isolated. With the conjunctival cell, only 1 of 12 trials was successful; several colonies of inositol-independent cells appeared about 2 weeks after onset of advanced degeneration of the parent culture. This variant continued to propagate in the inositol-free medium increasing by about 10-fold per week for about 6 months, after which it degenerated completely.

Influence of extent of dialysis of serum. Four sera were dialyzed against 40 times their volume of 0.85% NaCl with stirring at about 20°C for 2 hours and aliquots were further dialyzed against 8 more changes of 0.85% NaCl for 2 days at 4-10°C. These sera were tested for their ability to prevent degenerative changes of the inositol-requiring HeLa and conjunctival cells; both cells showed advanced degenerative changes whether serum dialyzed for 2 hours or 2 days was used. This is in agreement with the reported high concentration (1 to 4 μM) of inositol required by these cells(2,3). Three of these sera were analyzed for inositol. A sizable portion of serum inositol is not dialyzable and most of the dialyzable inositol is removed after 2 hours (Table II). Thus, the inositol-"free" medium contained about 0.5 μg of non-dialyzable inositol per ml which is presumably not utilized by the inositol-requiring cells; and, the inositol-"independent" cell may have be-

TABLE II. Total Inositol Content of Dialyzed Sera.

Serum	Total inositol, μg/ml		
	Pre-dialyzed	Dialyzed 2 hr	Dialyzed 2 days
Horse #1	8.4, 8.0*	5.2, 5.0	4.9, 5.3
Human (pool)	9.0, 9.0	not assayed	5.3, 5.0
" #1	8.8, 9.1	5.0, 4.1	3.7, 3.1

* Results of duplicate samples.

come independent of added inositol through its acquired property to use the non-dialyzable inositol.

Presence of vitamins in extensively dialyzed serum. The finding that about one-half of the serum inositol is not dialyzable prompted us to determine the presence of other vitamins in a dialyzed serum. A horse serum dialyzed against 10 changes of 10 times its volume of 0.85% NaCl at 0-10°C for 96 hours with constant stirring was assayed (Wisconsin Alumni Research Foundation) with the following results (expressed as amount per 100 ml dialyzed serum): 37 units Vit. A, 60 U.S.P. units Vit. D, 16 mg choline, 0.3 μg Vit. B_{12}, about 30 mμg biotin, 1.4 μg folic acid, 2 μg of pyridoxine, 58 μg pantothenic acid and 1.2 μg thiamine.

Lack of correlation between PPLO contamination and inositol requirement. Some cell strains are known to have been contaminated by pleuropneumonia-like organisms(8) without any associated signs of contamination such as cell degeneration, turbidity of medium and rapidly decreasing pH. It is perceivable that some of the nutritional studies reported previously may have been made with PPLO contaminated cells. It seems, therefore, desirable to determine if inositol requirement is related to PPLO contamination. Table III tabulates the status of PPLO contamination and inositol requirements of various cell strains; no relationship could be established. However, the effect of PPLO contamination on minimal concentration of inositol required cannot be determined from these data.

Discussion. The finding that strains of HeLa cell maintained independently in different laboratories differed in their inositol requirement seemed to explain satisfactorily the conflicting reports on the inositol requirement and the adequacy of Eagle's minimal growth medium. That such a difference should exist is not surprising since human cells in continuous cultivation resemble unicellular organisms(9) and since variability is an attribute of many unicellular organisms undergoing prolonged cultivation. Considering that the conditions for growth of these cells are not clearly defined, it is surprising that differences in the specific requirement for a growth factor between stock cultures of a human cell line have not been reported earlier. The ability of the inositol-requiring conjunctival cell, after being maintained in the 0.1 mM glucose medium which contains about 2 μg of inositol per ml, to propagate in the inositol-free medium deserves some emphasis. This suggests that the inositol-requiring culture acquired its inositol-independent property through a mechanism other than the deliber-

TABLE III. Lack of Correlation between PPLO Contamination and Inositol Requirement.

Cell strain*	Date tested	PPLO†	Inositol requirement
HeLa§ (Chang)	1956	+	Required
	1957	—	"
	1958	—	"
" (Roizman)	1957	—	Not required
	1958	—	" "
" (Eagle)	1957	+	Required
" #1 (Eagle)	1958	+	"
" #2 (Eagle)	1958	+	Inconclusive‡
" (Kunz)	1958	—	Required
" S3 (Barton)	1958	+	"
Conj.§ (wild)	1956	+	Required
	1957	—	"
	1958	—	"
" (.1 mM glucose)	1958	—	Not required

* Type 3 adenovirus produced characteristic cytopathogenic changes in all strains listed.

† — indicates failure to demonstrate PPLO by methods currently used (to be published by Dr. E. S. Murray). It is not certain when and where these cells were contaminated by PPLO prior to actual testing.

‡ No degeneration could be observed after 7 days in inositol-free medium; experiment was not continued because it is undesirable to work with PPLO contaminated cultures.

§ These cultures had been treated with 50 units of tetracycline/ml medium for about 2 wk around Jan. 1957 and have since been tested at regular intervals and found negative for PPLO contamination in presence of 50 units of penicillin and 50 μg of streptomycin/ml medium. Such treatment has not been regularly successful in eliminating PPLO from all cultures.

ate use of a defined selecting environment. The difficulty in isolating an inositol-independent culture through deliberate selection using an inositol-free medium tends to support this hypothesis. It is conceivable that unsuspected changes in the nutritional requirement of a cell culture could occur and thereby contribute toward further conflicting results. The role played by the serum protein component of the minimal growth medium is not known. A large number of substances, biologically active in low concentration are known to be present in dialyzed serum. Some of these substances are the enzymes, vitamins, hormones and trace metals. Thus, medium containing serum proteins should be considered non-chemically defined. Sera from various sources are known to influence cell propagation in a number of ways(4,10). The failure to demonstrate inositol as an essential for fresh explants of human amnion cell under the described experimental condition is also of interest. It suggests that caution is desirable in the generalization of data obtained through study with stable laboratory strains of human cells.

Summary. Under the described experimental conditions, qualitative differences in the inositol requirement of the following human cells have been found: 1) between several wild strains of HeLa cell obtained from several laboratories; 2) between a wild strain of conjunctival cell and a subculture propagating in a low glucose medium, and 3) between a stable laboratory strain and fresh explants of human amnion cells. The significance of these findings was discussed.

The author is grateful to Drs. J. C. Snyder and R. P. Geyer for their interest and suggestions in this work, and to Dr. E. S. Murray for his help in diagnosing PPLO contamination.

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Deposition of Fluoride in Soft Tissues Following Skeletal Saturation.* (24261)

MARTIN J. WAGNER,† GEORGE K. STOOKEY, AND JOSEPH C. MUHLER
Department of Chemistry, Indiana University, Bloomington and Indianapolis

The significant reductions in dental caries as a result of communal fluoridation can no longer be questioned. However, the biological effect of fluoride ingested at the level used to fortify fluoride-deficient water on tissues other than the dental structures has received little attention because: (1) determination of these micro-quantities of fluoride requires specialized analytical technics; and, (2) the almost immeasurably small quantities of fluoride found in tissues other than the teeth and skeleton had until recently been considered negligible. In addition, fluoride ingested at the level of 1 μg/ml is thought to be detoxified rapidly by excretion in the urine and incorporation into the skeleton. Recently, however, Muhler(1) and Buttner and Muhler(2) have shown that certain dietary factors (ascorbic acid and fats) increase fluoride retention both in the skeleton and in certain specialized soft tissues. It is important, then, to

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† U.S.P.H.S. Predoctoral Fellow of the N.I.D.R.

determine both distribution of fluoride in the skeleton and in the soft tissues when rats are given fluoride over prolonged periods of time, and to determine if fluoride is retained in soft tissues at a greater rate following skeletal saturation. This study was designed to investigate distribution of fluoride in the heart, liver, kidney, femur, sternum, and carcass, when rats are given amounts of fluoride considered optimal for children using water from communal fluoridation sources.

Materials and methods. A total of 93 weanling Sprague-Dawley strain rats were divided according to weight into 2 groups. Group I (52 animals) received exactly 1.0 mg F (as NaF) daily by stomach tube. Group II (41 animals) served as control and were not stomach-tubed. All animals were housed in large wire-screen cages, and were given fluorine-free drinking water and a low-fluoride (F = 0.5 μg/g) stock corn diet *ad libitum*.

At intervals of 15 days, 6 animals from each group were sacrificed. Both kidneys, the liver, heart, femur, and sternum were removed from each animal and analysed for fluorine by methods previously described(3). In preparation for analysis the tissues were placed in small silica dishes and dried in vacuum desiccators to constant weight. An accurately weighed 0.30 g sample of low-fluoride CaO was then added to each dish. the contents moistened with fluorine-free water, dried under infared lamps, and ashed in a muffle furnace for 6 hours at 600°C. Femurs were freed of soft tissue, charred under infared lamps, and similarly ashed at 600° for 6 hours.

After the desired tissues had been removed, the skinned carcasses were placed in silica dishes, charred over Meker burners, and ashed for 6 hours. For analysis, a homogeneous, powdered aliquot of femur, sternum, or carcass ash was placed directly in the perchloric acid distillation flask.

Results. Fig. 1 presents the results of fluoride analysis of the various tissues studied.‡ All of the soft tissues contained less than 3 μg total fluoride, even after ingestion of over 90,000 μg of fluoride. More significant is the fact that there were no trends suggesting that fluoride content would increase measurably with ingestion of additional fluoride.

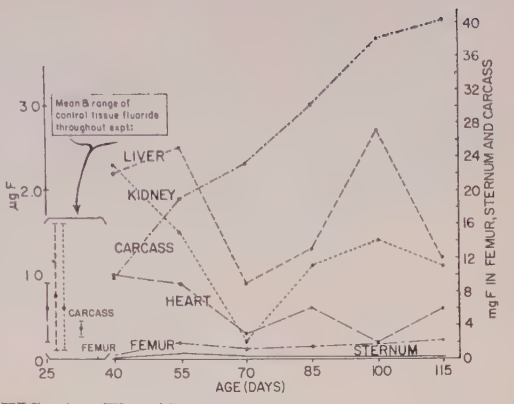


FIG. 1. Fluoride content of tissues of animals given 0 or 1 mg F daily and determined at 15-day intervals. (Each point represents mean of 6 animals.)

Fluoride content of the carcass increased markedly throughout the experiment, reaching a value of over 40 mg after 90 days of fluoride ingestion. The proportion retained, expressed as % of fluoride given, decreased from a maximum of 65% after 15 days of fluoride ingestion to an average 45% retention for the entire 90-day period.

The method of preparation of tissues for analysis was adopted after an extensive investigation was made of several procedures designed to (1 provide adequate fixation of fluoride during ashing; and (2 give a reproducible basis for calculation of fluoride concentration. Methods included (a) vacuum drying, (b) oven-drying, (c) homogenizing, and (d) freezing, all of which were followed by addition of calcium oxide and ashing. Procedure (a), used in this study, was found to be the most accurate and convenient for these purposes, as well as for satisfying the other requirements of the analytical procedure.

Some authorities(4,5) have advocated an elaboration of the general fluorine analysis procedure when undertaking analysis of tissues or blood which contain significant amounts of iron and other interfering substances. A standardization curve obtained on blood samples by adding known amounts of fluoride to small quantities of blood and sub-

‡ The numerical figures for fluoride content will be sent to anyone interested upon request.

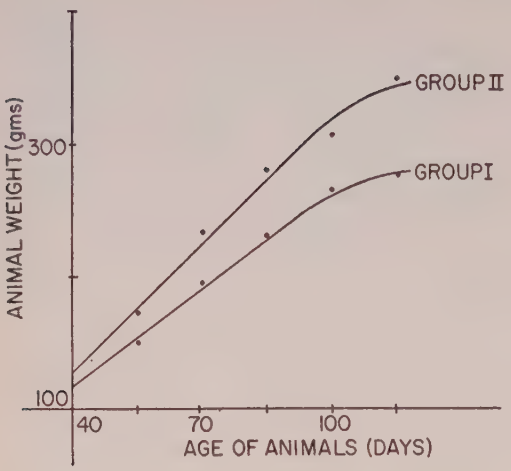


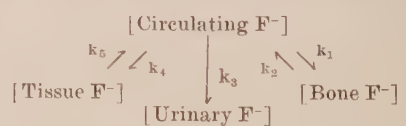
FIG. 2. Comparison of wt gain of animals given 1 mg F daily (Group I) with that of control animals (Group II).

jecting the ashed samples to analysis gave a curve which did not differ significantly from the curve obtained for analysis of pure fluoride solutions. The difference between the two curves was well within the experimental error of the general analysis curve for these amounts of fluoride. These results suggest that if extreme care is taken in determination of tissues with a low fluoride content, the standard analysis procedure can be used, particularly if other variables in the experiment are well-controlled.

Comparison of growth rates of the 2 groups of animals used in this study is presented in Fig. 2. The effect of fluoride at this concentration on weight gain is unmistakable. Decrease in weight gain in the fluoride group, however, has occurred with no significant increase in soft tissue fluoride content.

Discussion. Retention of fluoride in the carcass decreased noticeably after the period of rapid skeletal growth, at about 70 days of age, indicating that one factor in skeletal retention of fluoride is rapid turnover of the skeletal tissues during growth. Savchuck and Armstrong(6), and Miller and Phillips(7), reported that there was a greater uptake of fluoride by the femurs of young animals than with mature rats. Accretion of fluoride during rapid skeletal growth results from two mechanisms: (a) incorporation of fluoride into the tissues as they grow and increase in size; and (b) greater metabolic activity of the constituents of newly-formed bone, with accompanying greater deposition of fluoride. Perhaps a third factor, *i.e.*, absence of appreciable amounts of previously deposited fluoride, should also be considered.

Closely allied to this age-growth factor in its influence on fluoride retention is the intrinsic metabolic turnover rate of different tissues, as illustrated in the results of femur and sternum analysis. Concentration of fluoride in the femur was always greater than in the sternum, when based on ash weight. Fluoride deposition in bone of the same age may be dependent on vascularity of the bone, with consequent greater retention in bones having the higher blood supply. It has been assumed in the past that if soft tissues were to retain fluoride, this would begin to occur as the primary deposition site, the skeleton, approached fluoride saturation with the result that fluoride would not be as readily retained in the skeleton and would be deposited in the soft tissues. During the last 15 days of this 90 day study period only 14% of the ingested fluoride was retained by the skeleton compared to 65% retained initially. This suggests that saturation was being approached asymtotically. The final tissue fluoride values under these conditions were, however, well within the range of controls not receiving fluorine. Evidently fluoride not retained in the skeletal tissues was excreted rather than being secondarily deposited in vital tissues. This suggests that in the normal animal organism a situation favorable to the animal exists with regard to relative magnitudes of the equilibrium constants in the following equilibria:



Thus, when the organism has had little previous exposure to fluoride, resulting in a small Bone F^-, $k_1 > k_3 >> k_2$. However, as saturation of the skeleton is approached, k_2 may assume some magnitude, permitting a trend toward higher levels of circulating plasma fluoride. From these studies it would appear

that $k_3 > k_4$, so that labile fluoride is rapidly excreted. Nothing is known at present of the relative magnitudes of k_4 and k_5 or of the toxic consequences of a rapid turnover of fluoride through tissues in which only a trace amount may be found at any one time. Conceivably these equilibria could be altered by pathological conditions, particularly by renal disorders, which would decrease the constant k_3 in relation to k_4, or by endocrine dysfunction, causing increased turnover of skeletal constituents, an increase in k_2, with corresponding rise in level of circulating plasma fluoride. Investigation of these dynamic aspects of fluoride metabolism clearly is needed.

Summary. Administration of 1 mg F daily for 90 days to weanling rats failed to demonstrate any increase in fluoride content or concentration of heart, liver, or kidney, even as fluoride saturation of the skeleton was approached. Fluoride content of the carcass, femur, and sternum increased markedly with fluoride ingestion but rate of retention decreased throughout the experiment, particularly after attainment of skeletal maturity. Although no increase in soft tissue fluoride was demonstrated, the toxic effect of this level of fluoride (1 mg per rat per day) was demonstrated by the decreased weight gain of the fluoride animals when compared to the control group.

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Serum Gamma Globulin and *Trypanosoma cruzi* Agglutinin in Embryonic, "Normal" and Germ-Free Chickens.* (24262)

TIBOR BORSOS† AND H. NAIM KENT

Department of Pathobiology, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, Md.

The transmission of maternal serum proteins and antibodies to chick embryos *via* the yolk has been established by various investigators(1-3). The serum proteins of the developing embryos show progressive changes with age; Marschall and Deutsch(2) found low levels of gamma globulin in the serum of the 11-day embryo; later this serum fraction appeared in increasing amounts until after hatching. Brandly *et al.*(1) reported the absence of Newcastle virus antibody in the sera of 11-day embryos; they showed significant amounts of this antibody in the sera of 15-day-old, and high titers in the sera of 18-day-old embryos. Similar developmental patterns of *Trypanosoma cruzi* agglutinin in the sera of developing chick embryos was demonstrated by Borsos and Warren(4). It was also shown that germ-free chicks 86 days of age or older were devoid of detectable *T. cruzi* antibody. In contrast, all "normal" adult controls contained this factor in high titer. Recently, it was reported that the sera of 8 to 17-week-old germ-free chickens contained less gamma globulin than the "normal" controls; however, no difference was found between "normal" and germfree birds 4 to 8 weeks of age (5).

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† U. S. Public Health Service Research Fellow of the Nat. Cancer Inst. Present Address: Dept of Microbiology, Johns Hopkins School of Medicine, Baltimore, Md.

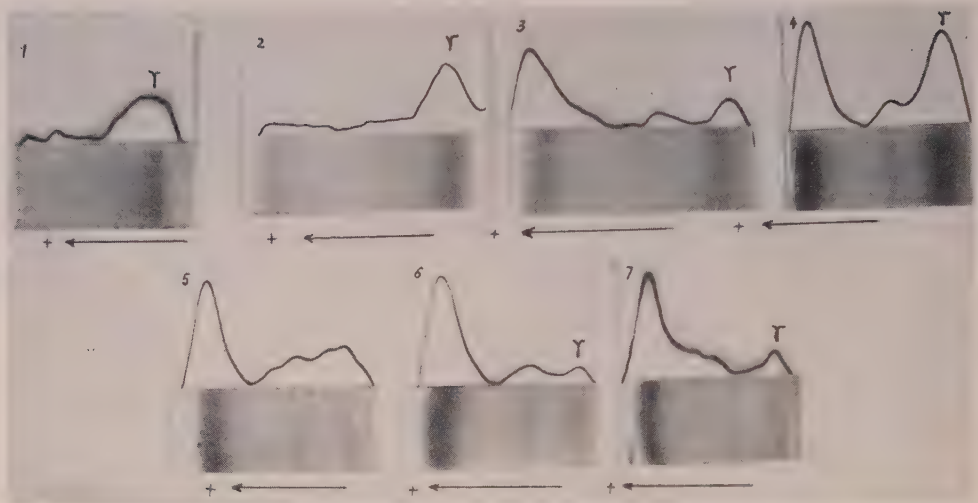

FIG. 1. Electrophoretic patterns of chicken sera. 1—15-day-old embryo. 2—2-day-old "normal" chick. 3—28-day-old "normal" chick. 4—"normal" adult. 5—24-day-old germ-free chick. 6—30-day-old germ-free chick. 7—313-day-old germ-free chick.

Considering these results, a correlation was expected between *T. cruzi* agglutinin and serum gamma globulin levels in chickens.

The purpose of this work was to study by paper electrophoresis the gamma globulin content of sera from developing embryos and from "normal" and germ-free chickens of different ages. Simultaneously, the presence of *T. cruzi* agglutinin in these sera was determined and correlated to gamma globulin content.

Materials and methods. The brasil strain of *T. cruzi* was maintained in culture as described by Warren(6). The technic for antibody assay will be described elsewhere in detail. Briefly, 1/10 dilutions of sera inactivated at 56°C for ½ hr. were tested against the culture forms of the organisms; the mixtures were sampled at time intervals and observed in the light microscope. 4+ denotes agglutination of all organisms, 0 denotes no visible agglutination at 90 minutes.

"Normal" sera were obtained from blood of laboratory hatched and reared White Leghorn chickens. The animals were bled by heart puncture or from the wing vein. The tubes with the clotted blood were stored usually overnight at 4°C; after centrifugation the sera were stored at –20°C.

Embryonic blood was obtained from a blood vessel of the chorioallantoic membrane and was handled similarly to adult chick sera(3).

Paper electrophoresis was performed in a cold room at 4°C with a Na-barbital-barbituric acid buffer at pH 8.6, ionic strength 0.1 for 5 hrs. under 400 volts and 10 milliamps (7).

After electrophoresis the papers were stained with brom-phenol blue. The protein patterns were photographed from the paper strips with an automatic recording photometer‡ and integrated. Since all the electrophoretic analyses were performed under the same conditions and the strips treated the same way, it is possible to compare the variations of gamma globulin levels to the adult "normal" sera.

Sera of germ-free chicks were generously supplied by Dr. T. G. Ward, Lobund Inst., Univ. of Notre Dame. They were shipped in dry ice and were stored at –20°C.

Results. A total of 46 sera were analyzed for gamma globulin content and *T. cruzi* agglutinating activity.

Fig. 1 presents a few typical electrophoretic patterns of sera from animals of different ages and rearing conditions. It is seen that the serum fractions vary extensively with the age of the donor bird. For this reason, in

‡ From Laboratories LERES, Paris, France.

TABLE I. Gamma Globulin Content and *T. cruzi* Agglutinin Indices of Chicken Sera.

	Embryonic														
Age, days	11	11	15	15	15	18	18	18							
% gamma g.	5	25	15	35	20	15	20	15							
Aggl. index	0	0	1+	1+	1+	2+	1+	2+							
	Young "normal"														
Age, days	2	2	2	5	5	5	22	22	22	22	22	28	28	28	28
% gamma g.	50	33	85	20	70	60	60	60	30	35	20	20	35	20	15
Aggl. index	3+ to 4+			n.d.	3+	4+	2+	2+	2+	1+	2+	1+	0	0	1+
	Young and adult germ-free														
Age, days	24	30	30	35	46	49	49	49	86	283	283	283	313	313	313
% gamma g.	30	20	10	30	15	30	30	110	35	10	15	5	15	15	30
Aggl. index	1+	1+	1+	1+	0	1+	0	3+	0	0	0	0	0	0	0
	Adult "normal"														
Age, days	180 to 500														
Area gamma g.	19	23	20	19	18	23	14	23	Avg area: 20*						
% gamma g.	95	115	100	95	90	115	70	115							
Aggl. index	4+	4+	4+	4+	4+	4+	4+	4+							

* Avg area of 20 was taken as 100% gamma globulin content.

comparing gamma globulin contents of the different sera, the amount of gamma globulin in each serum was expressed in terms of percentage of the average gamma globulin content of the 8 "normal" adult controls which was arbitrarily taken as 100%.

Table I records gamma globulin content of each serum together with the results of the *T. cruzi* agglutination tests. The data show that high gamma globulin contents were associated with high *T. cruzi* agglutinin indices. Sera of embryos were low in gamma globulin content; they also showed low agglutinating activity. Newly hatched birds contained gamma globulin levels up to the range of the "normal" adults; their agglutinin indices were correspondingly high.

The gamma globulin of the embryos and the newly hatched birds stemmed from the mother hen. By the 28th day after hatching gamma globulin levels (and agglutinating activity) dropped to low levels; in fact, some birds lost their agglutinating activity. After immunological maturity was reached all tested "normal" birds showed high gamma globulin contents with 4+ *T. cruzi* agglutinin indices. The gamma globulin in sera from germ-free birds continued to decline and stayed at low levels even in the adults. We have found one bird (age 49 days) which had a high gamma globulin level in its serum and also elevated *T. cruzi* agglutinin index. It is possible that this bird retained its maternal gamma globulin for an unusually long time after hatching; the alternative possibility of contamination must also be considered, although the bird tested free of contaminants at the time the serum was obtained (Personal communication, T. G. Ward.)

Conclusions and summary. The progressive changes in gamma globulin content in the sera of developing embryos indicated here confirm earlier findings(2). Following absorption of the yolk by the newly hatched chick, the transmitted gamma globulin levels decline until the young birds attain immunological maturity. The mature birds show high gamma globulin levels as illustrated by the electrophoretic patterns. In contrast, germ-free chicks reveal low levels of gamma globulin which remain so even at 313 days of age; *T. cruzi* agglutinin follows the same pattern. These results strongly suggest that a close correlation exists between serum gamma globulin levels and the indicator antibody. They also suggest that chickens reared under germ-free conditions, after losing the maternal antibody, do not replace it with gamma globulin of their own to any significant degree. Further studies are needed in order to follow the serum protein changes in older adult germ-free chickens and their response to controlled antigenic stimuli.

We are indebted to Drs. L. G. Warren and Rodrigo Zeledon for supplying the *T. cruzi* cultures used in this study.

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Susceptibility of Cortisone-treated Mice to Infection with Mouse Hepatitis Virus.* (24263)

THEODORE J. STARR AND MORRIS POLLARD

University of Texas Medical Branch, Department of Preventive Medicine and Public Health, Virus Research Laboratory, Galveston, Texas

The strain of mouse hepatitis virus (MHV) used has been described by Gledhill(1-3). It is the virus from which the enhancing agent, *Eperythrozoon coccoides,* has been excluded. This virus causes a mild, rarely fatal, infection in weanling Swiss mice and a fatal infection in suckling mice. In the latter case, focal necrotic lesions appear in the liver and evolve into diffuse, cirrhosis-like lesions. It was found that normally resistant weanlings became susceptible to fatal hepatitis infection when subjected to cortisone treatment. In this respect, cortisone has been used extensively to change the native resistance of various animals and embryonated eggs to infection by protozoan, mycotic, bacterial, rickettsial, and viral agents(4).

Materials and methods. Weanling Swiss mice, 8-10 g, and 1-2 day-old suckling mice, were used routinely. Stock virus was made from infected suckling-mouse livers as a 10% emulsion (w/v) in medium 199 or Hank's solution containing 0.2 mg/ml streptomycin and 200 units/ml penicillin. Liver preparations were clarified by centrifugation at 8,000 rpm for 20 min at 4°C, sealed in ampoules, and stored in a dry ice chest. The usual inoculum (0.05 ml) administered *via* the peritoneal route to weanling mice contained $10^{-3.5}$ suckling mice ID_{50}s(5) of MHV. Cortisone acetate (Upjohn Co., aqueous suspension, 25 mg/ml) was injected intramuscularly in single doses of 1.25 mg unless otherwise indicated. Mice were observed daily for 6 to 15 days. Survivors were killed with ether and autopsied. Livers showing lesions of questionable significance were homogenized in saline (10% w/v) and assayed in suckling mice.

Results. Large doses of cortisone alone caused some deaths, ascites, and distinct, pinpoint, whitish liver lesions in a small proportion of mice (Table I). In a series of 2 experiments, a total of 25 mice were given a single injection of cortisone (1.25 mg) and a total of 31 mice were given 4 daily injections of cortisone (total 5.0 mg). Observations were made daily for 12 and 15 days (Table I). Survivors were sacrificed and examined for evidence of liver damage and ascites. Of the mice which received 1.25 mg cortisone, an average of 8% died, none had liver lesions or ascites. Of the mice which received 5.0 mg cortisone, an average of 17% died, 9% had liver lesions, and 5% had ascites. Livers with cortisone-induced lesions were not infective for normal suckling mice. Several forms of unidentified bacteria were cultured in thioglycollate broth from these livers. Similar observations were reported by

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TABLE I. Effect of Cortisone on 8 to 10 g Swiss Mice Showing % Deaths, Liver Lesions, and Ascites.

Cortisone (mg)	No. of mice	Sacrificed (days)	Deaths	Lesions	Ascites
			%		
1.25	11	12	9	0	0
	14	12	7	0	0
5.00	11	12	9	18*	9
	20	15	25	0	0

* Homogenates of livers with cortisone-induced lesions were not infective for suckling mice.

others(6-9) and it is suggested that cortisone may activate a latent infection, transform nonpathogenic residents to pathogenic forms, and/or decrease host resistance to a point where normally nonpathogenic flora assume pathogenic roles.

The effect of single graded doses of cortisone on survival of weanling mice infected with MHV is shown in Fig. 1. One day after virus injection, cortisone was administered to 3 groups of 14 mice each as single graded doses of 0.3, 0.6, and 1.25 mg. A 4th group received only virus and a 5th group received only 1.25 mg cortisone on the 2nd day. There were no deaths in the virus control group after 12 days. In the cortisone control group, 7% of the mice died. There were 22, 57, and 85% deaths in the virus-infected groups which received 0.3, 0.6, and 1.25 mg cortisone, respectively. Cortisone reduced the innate resistance of mice to infection with MHV. Rate of mortality and number of deaths increased to a degree proportional to dosage of the steroid.

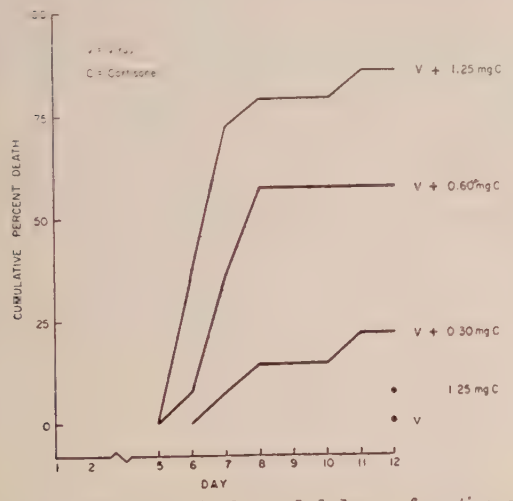

FIG. 1. Effect of single graded doses of cortisone on survival of mice infected with mouse hepatitis virus.

In another series of experiments, an attempt was made to determine the time interval between virus and cortisone injection which was most effective in decreasing host resistance. Five groups of mice were inoculated with virus on the 1st day. Following intervals of 1, 2, 3, and 5 days, 4 consecutive daily doses of cortisone (total 5.0 mg) were administered to each group of mice (Fig. 2).

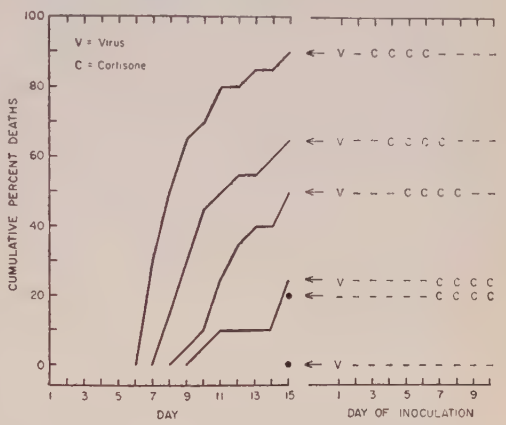

FIG. 2. Effect of cortisone on susceptibility of mice to hepatitis infection.

Deaths were not noted in the virus control group and cortisone alone accounted for 20% deaths. There was a definite decrease in rate of mortality as the time interval between virus and cortisone administration was increased from 1-5 days.

In other tests, cortisone and virus combinations were administered to 5 groups (I to V) of 18 mice each over a period of 5 days as shown in Table II. Deaths and liver lesions were noted on animals dying within 6 days at which time survivors were sacrificed. Table II shows the percentage of deaths incurred with and without liver lesions and the percentage of the survivors with liver lesions. Cortisone alone accounted for 6 and 22% deaths in cortisone-control groups IV (1.25 mg cortisone) and V (5.0 mg cortisone), respectively. However, 22% of the survivors of group IV had liver lesions. Comparison

TABLE II. Effect of Cortisone (C) and Virus (V) on 8-10 g Swiss Mice Showing % Deaths and % Survivors with Liver Lesions on the 6th Day.

Group	Day of inoc. 1	2	3	4	5	Deaths, no lesions	Deaths, with lesions	Survivors, with lesions
I	C	V	–	–	–	0	28	77
II	C	V	C	C	C	11	50	100
III	–	V	–	–	–	0	0	39
IV	C	–	–	–	–	6	0	22*
V	C	–	C	C	C	22	0	0

* Homogenates of livers with cortisone-induced lesions were not infective for suckling mice.

with the data in Table I shows that all of these survivors in group IV would have lived for at least 12 days. There were no deaths in virus control group III and 39% of the mice had liver lesions. As shown in Fig. 1 and 2, all of the mice in the virus control group III would have survived. Comparison of the percent deaths and percent liver lesions observed in groups I and II with their corresponding cortisone control groups IV and V again shows the effectiveness of cortisone in decreasing host resistance to infection with MHV (Table II).

Summary. A small proportion of mice inoculated with cortisone alone died or developed ascites and/or liver lesions. Bacteria were isolated from some of the livers showing cortisone-induced lesions. Homogenates of such livers were not infective for normal suckling Swiss mice. Cortisone acetate reduced susceptibility of normally resistant weanling Swiss mice to infection with the Gledhill strain of mouse hepatitis virus. The effectiveness of cortisone in decreasing host resistance was enhanced as dosage of the steroid was increased. There was a decrease in this cortisone effect as the time interval between virus and cortisone administration was increased from 1 to 5 days.

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Response of Germ-Free and Conventionally Reared Turkey Poults to Dietary Supplementation with Penicillin and Oleandomycin.* (24264)

M. FORBES,† W. C. SUPPLEE AND G. F. COMBS
(Introduced by P. Gyorgy)
Germ-free Animal Research Unit, Walter Reed Army Institute of Research, Washington, D. C., and Poultry Husbandry Department, University of Maryland, College Park

The mechanism of the growth-promoting effect of antibiotics in poultry rations has not been clearly established. The prevailing opinion seems to be that antibiotics act mainly by modifying the intestinal microflora(1-4). A basic question is whether antibiotics exert a growth-promoting effect in the absence of an intestinal microflora. Luckey(5) reported that no stimulation of growth was seen when germ-free chickens and turkeys were fed antibiotics at a level of 50 mg/kg diet. In subsequent studies Luckey, *et al.*(6) reported that

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† Dept. of Microbiology, School of Medicine, University of Pennsylvania. Present address: Temple University School of Medicine, Philadelphia.

lower levels of antibiotics (oxytetracycline 25 mg/kg, procaine penicillin 11 mg/kg) caused slight growth increments in germ-free chicks and that procaine penicillin (46 mg/kg) produced a statistically significant increase in the growth rate of germ-free poults. Recently Gordon, *et al.*(7), repeating Luckey's first studies, found no evidence of increased body weight in germ-free chicks fed procaine penicillin at a level of 50 mg/kg diet. These reports were based on comparisons of such limited numbers of birds that the significance of the observed differences is not clear. In more extensive experiments with germ-free and conventional chicks, it has been shown that the growth rate of chicks on an adequate diet is not affected by dietary supplements of penicillin (45 mg/kg) unless penicillin-sensitive organisms which depress growth are present (Forbes and Park, to be published). Experimental results were also obtained suggesting that *Clostridium welchii* type A is one such penicillin-sensitive growth depressing organisms(8, Lev and Forbes, to be published).

In the present paper a series of experiments with turkey poults is described in which the effect of dietary supplementation with procaine penicillin or oleandomycin was compared under germ-free and conventional rearing conditions.

Methods. Maryland Medium White poults hatched from eggs supplied by the Poultry Department, University of Maryland, and Beltsville White poults from eggs obtained from the Agricultural Research Center, Beltsville, Md., were used in these studies. The procedure followed in rearing germ-free birds§ was patterned after that of Reyniers *et al.*(9). Twenty-day embryonated eggs were immersed for 2 minutes in a detergent solution, then for 12 minutes in a 2% $HgCl_2$ solution maintained at 37°C, then were passed through a tube into a previously steam sterilized Reyniers germ-free unit where incubation was completed. Usually, 24 eggs were incubated in each unit and yielded 14 to 16 poults. The poults were weighed and distributed in 2 groups of 7 to 8 birds of approximately equal average weight. In each unit the 2 groups were kept in wire cages (approximately 11″ wide, 22″ long, 13″ high), one group serving as control while the other received the antibiotic supplemented diet.

§ In this report the term germ-free is intended to mean free of bacteria and fungi.

TABLE I. Basal Diet I.

	Per 100 g diet
Corn starch, g	41.40
Washed protein‡	44.44
Corn oil	4.04
DL methionine	.71
Glycine	.51
Choline chloride	.46
Diphenyl-p-phenylene-diamine	.0125
Minerals*	7.45
Vitamins†	.89

* Minerals, g/100 g diet (all c.p. or reagent grade) — 1.846 $CaCO_3$, 3.313 $Ca_3(PO_4)_2$, .808 K_2HPO_4, .455 Mg $SO_4 \cdot 7H_2O$, .606 NaCl, .051 $FeSO_4 \cdot 7H_2O$, .002 $CuSO_4 \cdot 5H_2O$, .031 $MnSO_4 \cdot H_2O$, .0002 $CoSO_4 \cdot 7H_2O$, .004 KI, .01 K AL (SO_4) 2.12 H_2O, .001 $Na_2MoO_4 \cdot 2H_2O$, .005 $Na_2SiO_3 \cdot 9H_2O$, .001 HBO_3, .012 $ZnCl_2$, .303 KCl.

† Vitamins, mg/100 g diet—12 alpha tocopherol acetate (from Myvamix), 2 Menadione, 60 thiamine, 10 riboflavin, 32 calcium pantothenate, 40 niacin, 6 pyridoxine HCl, .2 biotin, 2.4 folacin, .012 cyanocobalamin, 101 inositol, 1.0 p-aminobenzoic acid, 40 ascorbic acid. Also—5656 I.U. vitamin A (from Nopcay "20") and 529 I.C.U. vit. D (from Nopdex "15").

‡ Drackett C-1 Assay protein, washed once with tap water and 3 times with distilled water at pH 4.6, then dried at 50-60°C.

Eggs used for the conventional (non germ-free) tests were similarly sterilized but were transferred from the $HgCl_2$ solution into a standard incubator. After hatching the birds were transported to the Univ. of Maryland and distributed in electrically heated wire floored battery brooders. Lights were left on continuously both in the germ-free units and in the room where the conventional tests were carried out. The temperature inside the germ-free unit was 37-38°C when the birds hatched, being gradually decreased to 25-27°C over a period of 10 days and maintained there until the end of the experiment. Food and water were supplied *ad libitum*. Individual weights were taken initially and after 7 and 14 days on test.

Diets. The composition of the purified type washed Drackett protein starch basal diet is shown in Table I. All feed was sterilized by placing one kg portions in gauze bags in a layer not exceeding one inch in thickness in the autoclave of each germ-free unit.

Steam was allowed to flow through the autoclave for 10 minutes, the pressure was then increased to 17 p.s.i. (252-255° F), maintained for 25 minutes and gradually returned to normal. The feed was then either transferred into the germ-free unit or removed for use in the concurrent tests with conventionally reared poults.

Weighed quantities of procaine penicillin G or oleandomycin (Pfizer) mixed with starch were sealed in glass ampules, sterilized by irradiation, introduced into the germ-free unit *via* a germicidal trap and mixed into the sterile diet. Similarly sterilized antibiotics were used in the conventional tests. Irradiation‡ (1,800,000 rep) did not modify antibacterial activity of the antibiotics as measured by the tube dilution method using *Micrococcus pyogenes var. aureus H* as the test organism.

Sterility tests. Moistened swabs were used weekly to take multiple anal samples or fresh fecal specimens from all animals. The swabs were inoculated in thioglycollate broth, in cooked meat medium and in trypticase soy broth. Other swabs were streaked on brain-heart infusion agar containing 5% blood, on lactobacillus selection agar (Baltimore Biological Laboratory) and on Sabouraud agar. Replicate cultures were incubated at room temperature and at 37°C. Some blood plates were incubated anaerobically at 37°C. Cultures were kept for 2 weeks and examined periodically. Gram stains were made of samples of the broth cultures. At the conclusion of most of the experiments, a bird was killed inside the unit and samples of the intestinal contents were cultured in the various media.

Statistical analysis. The growth data were subjected to the Fisher F test, results from replicate tests being combined. To simplify the calculations, the numbers in the various comparisons were equalized by selection using a table of random numbers. No discrepancy was found between total data as compared to selected data.

‡ Performed through the courtesy of Dr. Howard Andrews, Radiation Branch, Nat. Cancer Inst. and Mr. John Hickey, Sanitary Engineering Branch, N.I.H., Bethesda, Md.

TABLE II. Effect of Procaine Penicillin (45 mg/kg) on Growth of Germ-Free and Conventional Poults.

		Avg wt in g on 14th day				
		No supplement		Penicillin		
Exp.	Status	No. birds	Wt	No. birds	Wt	Diff.
1	GF	6	221	7	198	−23
	Conv.	10	182	10	212	+30
2	GF	7	217	7	227	+10
	Conv.	12	181	15	219	+38
3	GF	10	179	6	186	+ 7
	Conv.	11	138	9	192	+54
1, 2 & 3 combined	GF	23	202	20	207	+ 5
	Conv.	33	170	34	212	+42

TABLE IIA. Statistical Analysis of Randomly Selected Data of Combined Experiments Shown in Table II.

	Avg wt in g on 14th day (20 birds/group)		
Status	No supplement	Penicillin	Diff.
GF	201	207	+ 6
Conv.	166	207	+41

Analysis of Variance

		Source of var.		
Main effects	DF	MS	F	p
Status: GF *vs* conv.	1	6,187	1+	n.s.
Treatment: Pen. *vs* no pen.	1	10,732	1+	"
Interaction Treatment *vs* status	1	5,993	5.50	<.05
Error	76	1,089		

Results. The conventionally reared poults fed the penicillin supplemented ration gained an average of 42 g more than their controls receiving the unsupplemented ration (Table II). In contrast, the germ-free birds fed the basal diet grew at a rate comparable to the conventional birds receiving penicillin and growth was not improved by addition of penicillin. Statistical treatment of the data (Table IIA) indicates that response to penicillin in the conventional environment was significant ($p<0.05$).

Oleandomycin also caused a marked growth improvement in the conventional poults but none in the germ-free birds (Table III, IIIA). As with penicillin, supplementation with oleandomycin increased growth in the conventionally reared birds to a level comparable to

TABLE III. Effect of Oleandomycin (30 mg/kg) on Growth of Germ-Free and Conventional Poults.

		Avg wt in g on 14th day				
		No supplement		Oleando		
Exp.	Status	No. birds	Wt	No. birds	Wt	Diff.
1	GF	7	229	7	258	+29
	Conv.	9	171	7	193	+22
2	GF	8	219	7	193	−26
	Conv.	19	175	19	226	+51
3	GF	12	173	11	176	+ 3
	Conv.	9	159	10	179	+20
1, 2 & 3 combined	GF	27	201	25	199	− 2
	Conv.	37	170	36	207	+37

TABLE IIIA. Satistical Analysis of Randomly Selected Data of Combined Experiments Shown in Table III.

	Avg wt in g on 14th day (25 birds/group)		
Status	No supplement	Oleando	Diff.
GF	199	199	0
Conv.	178	208	+30

Analysis of Variance

		Source of var.		
Main effects	DF	MS	F	p
Status: GF *vs* Conv.	1	1,584	1	n.s.
Treatment: Oleando *vs* no oleando	1	7.856	1	"
Interaction				
Treatment *vs* status	1	13,537	8.47	<.01
Error	96	1,599		

that of the germ-free birds.

The results of these experiments are in agreement with the theory that growth response to antibiotics is due to their action on the microflora.

Summary. Maryland Medium White and Beltsville White poults reared under germ-free conditions showed excellent growth on a purified type washed Drackett protein starch basal diet. Supplementation of the diet with procaine penicillin (45 mg/kg) or with oleandomycin (30 mg/kg) did not improve growth. In concurrent tests, conventionally reared poults fed the basal ration grew at a lower rate than the germ-free birds while those fed the basal ration supplemented by either penicillin or oleandomycin grew at a rate comparable to that of the germ-free birds.

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Influence of Long Term Fat-Feeding on Excretion of Cholesterol-4-C^{14} Metabolites.* (24265)

JEAN D. WILSON AND MARVIN D. SIPERSTEIN
(Introduced by Donald W. Seldin)
Department of Internal Medicine, The University of Texas Southwestern Medical School, Dallas

Studies in this laboratory have recently been concerned with the influence of variations in the type and quantity of dietary fat on metabolism of cholesterol-4-C^{14}. We have previously reported that a 4-day period of feeding rats isocaloric diets containing corn oil, as compared to diets containing lard or no

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TABLE I. Fecal Excretion of Cholesterol-4-C^{14} after 72 Days of Feeding Fat-Free, Corn Oil or Lard Diets.

Group	Rat #	Diet: Fat content	Diet: Avg daily intake g	Diet: Avg daily intake Cal.	Wt (g) at Begin.	Wt (g) at End	Avg daily dry wt of feces (g)	% administered dose recovered as: Neutral sterols: Non-dig. precip.	Neutral sterols: Dig. precip.	Bile acids	Total recovery
1	1	None	19.3	71.8	182	354	1.29	1.43	9.00	14.82	25.25
	2	30% corn oil	12.7	66.8	175	284	.92	20.21	9.81	22.46	52.48
	3	30% lard	12.1	63.6	173	337	.86	1.17	14.71	28.63	44.51
2	4	None	17.9	66.6	169	284	1.12	1.34	11.06	12.88	25.28
	5	30% corn oil	12.5	65.8	191	315	.92	21.96	8.43	33.80	64.19
	6	30% lard	12.4	65.2	154	273	.86	7.00	13.69	38.80	59.49
3	7	None	15.3	56.9	148	259	1.01	1.08	11.59	13.56	26.23
	8	30% corn oil	11.1	58.4	174	325	.90	17.77	7.43	28.49	53.69
	9	30% lard	10.9	57.3	172	288	1.06	6.60	21.73	34.89	63.22
4	10	None	18.4	68.4	195	333	1.21	1.95	7.33	20.51	29.79
	11	30% corn oil	12.6	66.3	192	300	.81	25.08	11.11	23.34	59.53
	12	30% lard	12.9	67.9	166	330	.89	3.66	16.28	35.70	55.64

fat, resulted in marked acceleration in metabolism of cholesterol-4-C^{14} to *non-digitonin precipitable* neutral sterols(1,2). The primary purpose of the present series of experiments was to examine the excretory products of cholesterol-4-C^{14} in rats subjected to long term feeding of diets containing no fat, 30% lard or 30% corn oil. Initial attempts to characterize the non-digitonin precipitable neutral sterol fraction in the feces of these animals are also reported.

Materials and methods. Male rats of the Long-Evans strain, weighing 160-195 g were pair-fed isocaloric quantities of diets containing no fat, 30% corn oil, or 30% lard† for a period of 72 days. They were then anesthetized with ethyl ether, and a solution of cholesterol-4-C^{14}, containing 1.12 x 10^6 cpm and prepared with the aid of Tween 20(3), was injected into the tail vein. Feces were collected at intervals for 14 days.

The samples of feces were dried and extracted continuously for 24 hours with ethanol in Soxhlet extractors. The methods for separating neutral sterols and bile acids have been described(2). In addition, the supernatant portions and the washes which remained following precipitation of the sterol digitonides were combined, taken to dryness, and redissolved in acetone-ethanol. Digitonides were again precipitated from this fraction by the method of Sperry and Webb(4) in order to assure that all digitonin precipitable sterols had been removed.

The supernatant fractions and washes which remained after digitonin precipitation of the neutral sterols from feces of rats fed the experiment diets for short periods (2), were combined, taken to dryness, and dissolved in pyridine. Excess digitonin was precipitated in ethyl ether(5), and the supernatant was again taken to dryness. Ketonic and non-ketonic fractions of this non-digitonin precipitable residue were separated by precipitation with Girard's Reagent "T" as follows(6): The residue was dissolved in 10% acetic acid in ethanol and refluxed for 30 minutes in the presence of an excess of Girard's Reagent "T". This solution was brought to pH 6 with a cold $NaHCO_3$ solution and extracted 3 times with ethyl ether. The bicarbonate-acetic acid solution was then acidified with 10 N HCl and extracted 3 times with ethyl ether. Each ethyl ether fraction was then taken to dryness, dissolved in methyl alcohol, added to 0.4% diphenyloxazole in toluene and assayed for C^{14} in a liquid scintillation counter.

After assaying for C^{14} in these experiments, an internal standard of cholesterol-C^{14} was added to each sample. The C^{14} of the standard was determined, and, when necessary, corrections for quenching were made.

Results. In Table I are listed the results

† The composition of the diet has been described (2).

TABLE II. Distribution of Non-Digitonin-Precipitable Sterol-C^{14} between Ketonic and Non-Ketonic Fractions.

Group	No. of rats	Diet	% administered dose recovered as non-digitonin precipitable neutral sterols	% non-digitonin precipitable sterols in	
				Ketonic fraction	Non-ketonic fraction
1	4	Fat free	9.61 ± 1.82	8.46 ± 4.68	91.54 ± 5.62
2	4	30% corn oil	34.75 ± 2.42	6.62 ± 4.02	93.38 ± 4.03
3	4	30% lard	11.64 ± 3.68	8.82 ± 5.25	91.18 ± 5.27

from pair-fed rats receiving diets containing no fat, 30% corn oil or 30% lard for 72 days. Average daily caloric intake of the various rats was similar, and there was no difference in weight gain of the animals fed the various diets. (Average weight gain was 134 g for the animals fed fat-free diets, 123 g for the animals fed corn oil, and 141 g for the lard group). Furthermore, average daily dry weights of the feces were comparable.

Rats fed the corn oil or lard diets uniformly excreted more C^{14} than did rats fed diets containing no fat. Excretion of bile acid-C^{14} and digitonin precipitable neutral sterol-C^{14} tended to be higher in the rats fed lard. As in the short term experiments, however, the most striking difference among the various animals was the marked increase in excretion of non-digitonin precipitable neutral sterols in the rats fed corn oil. This accounted in these rats for 18-25% of the administered dose of C^{14}.

The results of preliminary attempts to identify the nature of the non-digitonin precipitable neutral sterols in previously reported experiments on rats fed the experimental diets for 4 days(2) are listed in Table II. Ketonic sterols were separated by Girard's Reagent "T" precipitation, and radioactivity of both ketonic and non-ketonic fractions was assayed. It is evident from these studies that the marked acceleration in excretion of non-digitonin precipitable neutral sterols in rats fed corn oil is accounted for primarily by the C^{14} recovered in the non-ketonic sterol fraction.

Discussion. The mechanisms by which dietary fat influences cholesterol metabolism both in man(7) and rat(8) remain unknown. The previous finding of a qualitative difference in metabolism of cholesterol-4-C^{14} in rats fed either corn oil or lard has suggested that, in part, the effect of unsaturated fats may be secondary to acceleration of excretion of non-digitonin precipitable neutral sterols(1,2). This concept is supported by the finding of Swell, Trout, Hopper, Field and Treadwell (9) that as much as 20% of an injected dose of cholesterol-4-C^{14} may be converted to non-saponifiable, non-digitonin precipitable sterols within 24 hours in rats fed oleic acid. The present studies in rats fed for 2 months diets which varied in fat content confirms the finding that isocaloric substitution of 30% corn oil diet for either fat-free or 30% lard diets results in marked acceleration in excretion of non-digitonin precipitable neutral sterol-C^{14}, accounting in these rats for 33-42% of excreted C^{14}. Furthermore, this alteration in cholesterol-C^{14} metabolism is no more pronounced after 72 days than after 4 days of corn oil feeding(2). As yet neither the site of transformation nor the nature of this non-digitonin precipitable neutral sterol is known. Since no 3-alpha-hydroxy neutral sterols have yet been isolated in nature, it was thought that excretion of Δ^4-cholestene-3-one or cholestane-3-one might account under these circumstances for the non-digitonin precipitable fraction. However, these findings clearly demonstrate that the ratio of carbonyl to non-carbonyl fractions in the non-digitonin precipitable neutral sterols is unchanged by feeding of corn oil, indicating that ketonic sterols can account for only a negligible portion of the non-digitonin precipitable sterols. Thus, the sterol, which is excreted in increased quantities under the influence of corn oil feeding, is presumably a 3-alpha-hydroxy neutral sterol, the nature of which remains to be determined.

Summary. Effects of long-term feeding of various types and amounts of fat on fecal cholesterol excretion are reported. Diets con-

taining 30% corn oil produce marked acceleration in excretion of C^{14} as non-digitonin precipitable neutral sterols in rat feces, accounting in these animals for 17-25% of the administered C^{14}. This non-digitonin precipitable neutral sterol does not contain a ketonic group and is presumably a 3-alpha-hydroxy sterol.

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Characteristics of the Asian Strain of Influenza A.* (24266)

WILLIAM J. MOGABGAB, WILLIAM PELON, GEORGE E. BURCH AND BARBARA HOLMES
Department of Medicine, Tulane University School of Medicine, New Orleans, La.

Besides the marked antigenic variation found in the Asian strain of influenza A, several other characteristics differentiate it from influenza A viruses isolated in recent years (1,2). Included were growth behavior in the embryonated egg, and reaction with various erythrocytes, with antibody and with non-specific inhibitors. Although the virus propagated in monkey kidney cultures tended to minimize such differences, even in this case hemagglutination behavior set it apart from earlier strains. Pertinent features of Asian virus isolated and propagated in monkey kidney cultures and in the chick embryo are here described.

Materials and methods. Cultures (TC) were prepared with 0.25% trypsin (Difco, 1-250) digested Rhesus monkey kidneys, grown at 35°C in stationary tubes with 0.5% lactalbumin hydrolysate (Nutritional Biochemicals) and 2% calf serum (Microbiological Associates) in Earle's solution. After 7-12 days they were washed and maintained at 35°C with Mixture 199 (Microbiological Associates), and pH held between 7.0 and 7.6 during experiments by adding $NaHCO_3$ solution or allowing escape of CO_2. Ten day White Leghorn embryonated eggs were used for virus isolation in the amniotic sac or propagation in the allantoic sac (E). Throat garglings were obtained with Mixture 199, 0.5% lactalbumin hydrolysate in Hanks' solution or boiled skimmed milk and were collected from July through Dec. 1957 in Louisiana. Except where references are made to observations on several strains, experiments were done with Asian virus isolated in New Orleans from a Boy Scout on board a train from the 1957 Jamboree, Valley Forge, Pa. Separate passage lines were derived from the original specimen and maintained in the egg and in TC. The egg-ferret-mouse-egg line ($E_4F_1M_3E_{12}$) of A/Japan/305/57 (FE) was obtained from the Communicable Disease Center, U.S. Pub. Health Serv. Erythrocyte (rbc) suspensions for hemagglutination (Ha) were based on packed cell volume in a 15 ml conical tube after centrifugation in an International No. 1 at 1500 rpm for 10 minutes. A final concen-

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TABLE I. Virus Isolations in Egg and in Tissue Culture (TC) from Specimens Obtained from Serologically Diagnosed Cases.*

Isolation system	No. cases	No. isolations	% isolations
Egg	100	62	62.0
TC (7 day)	64	10	15.6

* Four-fold or greater increments in hemagglutination-inhibition were obtained in paired sera using the monkey kidney line of Asian virus and RDE treated sera.

tration of 0.25% rbc was used in all experiments. Receptor destroying enzyme (rde) was prepared from filtrates of *Vibrio cholerae* cultured at room temperature on a shaking apparatus. For hemagglutination-inhibition (HaI) titrations sera were treated overnight with rde or M/90 potassium periodate, or heated at 56°C for 30 minutes. Titers were recorded as initial dilution of antiserum inhibiting agglutination of 0.25% human type O rbc by 4 Ha units of virus. Neutralization titers were determined with sera inactivated at 56°C for 30 minutes and read as the initial dilution of serum inhibiting growth of 10^2TCID_{50} at 7 days or 10^2EID_{50} at 3 days.

Primary isolation. A larger percentage of virus isolations was made in the amniotic sac even on first passage than in TC (Tables I, II). Second passage was not used in TC since previous results with influenza B isolations had suggested that a medium change on day 3-4 accomplished as much(3). In contrast to that virus, 10 to 100 times as much Asian strain was required to infect TC as the amniotic sac. Although the percentage of isolations of Asian virus was slightly less than might have been expected with influenza A strains in the chick embryo, the results in TC were not unlike those with previous strains of this type of virus(4). Serologic identification of hemagglutinins in amniotic fluid was sometimes difficult because of low titers or lack of combination with antibody (Q phase) until additional passages had been made(5). The latter characteristic was not encountered in strains isolated in TC. Perhaps the poor results shown in Table II with the hemadsorption technic might have been improved by use of an intermediate medium change to remove inhibitory substances(6). Although hemadsorption was satisfactory for detection of virus growth with a TC line of Asian virus, a conventional hemagglutination pattern could also be observed in positive tubes.

TABLE II. Results of Inoculation of Egg Positive Specimens in Egg and in Monkey Kidney Cultures.

Isolation system	No. specimens	No. positive	% positive
Egg*—E_1		33	43.4
E_2		37	48.7
Q (E_3–E_7)		6	7.9
Total	76	76	100.0
TC (7 day)†	51	10	19.6
TC (HaAds)‡	27§	3	11.1

* More than 2 amniotic passages (E_1 and E_2) were done if hemagglutinins were not inhibited by specific antibody (Q phase).

† Fluids were replaced at 3 and 7 days and hemagglutinating titers determined.

‡ Human type O RBC were added at 1-2 days for hemagglutination-adsorption.

§ 9 were also positive in TC (7 day).

Hemagglutination. Titers of agglutination of various rbc by Asian virus as shown in Table III resembled influenza B strains(7,8).

TABLE III. Titers of Agglutination of RBC from Various Species by TC and E Lines of Asian Virus.

RBC, .25%	Temp., °C	TC_{2-4} (MK) Active	TC_{2-4} (MK) Inactive*	E_{3-5} Active	E_{3-5} Inactive*
Human	4	64	32	256	64
	24	64	32	256	64
Fowl	4	64	32	512	256
	24	64	32	512	128
Sheep	4	16	8	64	32
	24	8	4	64	32
Monkey	4	32	16	512	64
	24	32	8	256	64

* Viruses were inactivated by heating at 56°C for 30 min.

Absent were the lower titers with fowl rbc and lack of agglutination of sheep rbc at 24°C characteristic of early egg passages of previous influenza A strains. Differences between the TC and E lines of Asian virus were manifest only by lesser affinity of the TC line with sheep rbc and disagglutination was more rapid and extensive especially with the latter as well as fowl rbc. This resembled the 1953 strain of influenza A in which passages in the egg increased reactivity with fowl and sheep rbc receptors to a greater degree than pas-

sages in human kidney cultures. Adaptation of the 1953 TC line to monkey kidney cultures also resulted in equivalent titers with human, fowl or monkey rbc and lesser affinity for sheep rbc(4,8).

Growth of virus. Although Asian virus titers in the amniotic or allantoic sacs were considerably lower than those observed with the 1953 strain of influenza A, growth in monkey kidney cultures was similar to the latter virus(4). Differences in isolated strains were reflected by variability in virus titers obtained on early amniotic passages, some requiring several to equal titers obtained with others upon initial inoculation. After adaptation to the allantoic sac an incubation period of 2 days was adequate for determination of infectivity (EID_{50}). Asian virus often killed chick embryos inoculated in the amniotic sac and produced relatively marked cytopathogenic effects in monkey kidney cultures. Strains isolated from human lung autopsy specimens demonstrated no distinctive features in the egg or TC.

Serum inhibitors. Agglutination of human type O rbc by the egg line of Asian virus, either active or heated at 56°C for 30 minutes, was not inhibited by normal human, rabbit or fowl sera until more than 4 passages had been made. The TC line, in contrast, was quite sensitive to non-specific substances, and heating human or fowl sera at 56°C for 30 minutes increased degree of inhibition by fourfold or greater. Additional passages in the allantoic sac resulted in similar inhibition of the E line with human and rabbit, but not fowl, sera. The EFME line of Asian virus behaved much like the TC line, but periodate was required for inactivation of inhibitor rather than rde, which was adequate for the latter. Treatment of serum at 35°C overnight with equal quantities of 1.6% trypsin (9) removed inhibitors of hemagglutination in all instances except with rabbit serum and the TC and EFME lines. Temperatures of 65°C for a half hour did not reduce inhibitor titers.

Reaction with antibody. Early egg passage Asian virus reacted weakly with HaI antibody in rde treated sera. In fact, diagnostic, 4-fold or greater, increments in titer were found in only half the acute-convalescent serum pairs that were positive with TC virus. With the TC line diagnostic HaI antibody increments were found in all cases in which virus had been isolated or antibody increase demonstrated by complement-fixation with egg propagated virus. Increased reactivity of the E_4 virus resulted when sera treated with heat alone were used. The same was accomplished by additional egg or intermediate animal passages (EFME), but it became necessary to remove non-specific inhibitors in sera with both the latter. Antibody titers in convalescent sera from influenza cases were higher when determined with the TC than any of the E lines (Table IV), either because of a closer antigenic relation to the infecting virus or due to greater reactivity of the TC line with antibody. On the other hand antibody titers in post-vaccinal sera were similar with the TC or EFME lines(10,11). It was unreasonable to assume that the lower HaI antibody titers with E_4 and rde treated sera were due to destruction of antibody since this did not occur with the E_7 and TC lines. Although it seemed likely that an additional serum component was participating in the inhibition of E_4 by untreated convalescent serum, titers were not increased by addition of fresh normal serum or calcium to rde treated serum. Lower titer antibody increments were often not demonstrable in paired sera from influenza cases regardless of treatment of sera when tested with the E lines. Even with the TC virus and a measurable 4-fold antibody increase the convalescent serum titer was sometimes 1-4. This indicated little previous antigenic experience with influenza A, and also suggested limits to

TABLE IV. Mean Hemagglutination-Inhibition Antibody Titers Determined with TC and E Lines of Asian Virus.*

	Virus				
	TC_{2-4}	E_4	E_4	E_7	EFME
Treatment of serum	RDE	△56°C	RDE	RDE	Periodate
Acute serum titer	<4	<4	<4	<4	<4
Convalescent serum titer	120	37	16	32	46

* Geometric mean titers determined from 10 paired sera.

TABLE V. Mean Neutralization Titers Determined with TC and E Lines of Asian Virus.*

	Virus			
	TC_{2-4}	E_4	E_6	EFME
Host system	TC	Egg	Egg	Egg
Acute serum titer	<4	<4	<4	<4
Convalescent serum titer	91	4	4	10

* 10^2 ID_{50} of virus was added to dilutions of heat inactivated sera. Results are geometric means from 6 pairs of sera from influenza cases.

sensitivity and a threshold of the technic of determining HaI antibody.

Avidity of the E line for neutralizing antibody determined in the allantoic sac appeared slight as shown in Table V. Increasing the temperature from 4° to 37°C and the time from 30 to 60 minutes did not change the combining capacity of the E line with neutralizing antibody. Reduction in amount of virus by 10-fold also did not greatly increase titers as compared to those obtained in TC. Quantitatively, the results of antibody determinations in the latter system were similar to those of HaI with TC virus and rde treated sera. It appeared that the E line had more affinity for neutralizing antibody in post-vaccinal rather than post-influenzal sera, again suggesting its lack of close relationship to the natural virus(11).

Although complement-fixation was necessary to establish relationship of Asian virus to other influenza A strains, some evidence could be obtained by HaI. Antibody increments in the order of 2-fold were not uncommon in paired sera from Asian influenza cases when tested with the 1953 Great Lakes or 1957 Denver influenza A viruses. Also, the TC line of Asian virus was inhibited at low titers with Denver rooster antiserum, although not with Great Lakes rabbit antiserum.

Discussion. Features of Asian virus characteristic of influenza A as a group in addition to antigenic relationship were behavior upon primary isolation in TC and the egg and rate of growth of a minimal infecting dose in the allantoic sac. Thus, the proportion of cases from which virus could be isolated in the amniotic sac was at least 60% and was as much as 80-90% when specimens were carefully collected early in the illness. In addition, primary isolation in TC required 10-100 times as much virus as the chick embryo in contrast to results with influenza B(3). Also, an incubation period of 2 days was adequate for determination of infectivity with adapted virus in the allantoic sac, while influenza B required 3 days(12).

Among the differences between the Asian and other influenza A strains reactivity with receptors was of particular interest. The lack of normal serum inhibition of early egg passages of the strain of Asian virus described herein was reminiscent of similar behavior of the human TC line of a 1953 influenza A with non-specific inhibitors(8). Although the monkey kidney culture line of Asian virus was quite sensitive to normal serum inhibition, additional passage of the 1953 human TC line in monkey kidney cultures also produced a similar change(4). Accordingly, it is possible that lack of sensitivity to inhibitors was a feature of the natural virus. Support for the contention that such a characteristic might facilitate spread was obtained by demonstration of an inhibitor of infectivity of the monkey kidney line of the 1953 virus in normal heat inactivated rabbit sera, and this inhibitor was removable by rde(4). Affinity for receptors of fowl and sheep erythrocytes was another unexpected attribute of the Asian strain. Since this was found even on initial passages in the egg or TC it is unlikely that this was caused by the laboratory host system. Nevertheless, studies with Asian virus in human tissue culture might serve to answer some of these questions. Surprisingly in this regard it has not yet been possible to isolate or propagate virus in human embryo kidney cultures from 6 original specimens that had previously been positive both in the egg and monkey kidney cultures. This occurred despite two passages as well as several fluid changes in cultures that supported growth of the human tissue culture line of the 1953 influenza A line (4).

The occurrence of strains that were initially weak reactors with antibody (Q phase) was of especial interest in a pandemic virus even though this has been previously described with epidemic strains of influenza A (5). Al-

though this might have been caused by growth in the chick embryo, since similar characteristics were not observed in the TC isolates, it is also reasonable to assume that preferential growth of Asian virus in the egg allowed retention of certain natural characteristics longer than TC. Probably the same mechanism accounted for lack of reactivity with inhibitors and antibody and might be of consequence in explaining spread of the virus if it occurred during infection of man. One might speculate that virus propagated in TC was antigenically closer to the natural agent, but that grown in the egg was reactively more similar.

Summary. The amniotic sac of the chick embryo was much more effective than monkey kidney cultures for isolation of the Asian strain of influenza A, and virus was obtained from at least 60% of serologically diagnosed illnesses. Both monkey kidney culture and chick embryo propagated virus agglutinated human, fowl, sheep and monkey erythrocytes at 4° or 24°C, but titers were lower with sheep red blood cells. Asian virus multiplied readily in monkey kidney cultures, but several passages were required for adaptation to the allantoic sac. Some strains during early egg passages were not inhibited by non-specific substances in normal sera, and some were initially non-reactive with antibody, especially in rde treated sera. Increased sensitivity to inhibitors and antibody occurred with additional chick embryo passages, but remained lesser than the tissue culture line. Asian virus propagated in monkey kidney cultures was preferable to the egg line for determinations of hemagglutination-inhibition and neutralizing antibody by virtue of the higher titers, comparability of response and reproducibility.

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Antibody Response to Asian Influenza Vaccination in Man.* (24267)

WILLIAM PELON, WILLIAM J. MOGABGAB, LAWRENCE DIETLEIN, GEORGE E. BURCH AND BARBARA HOLMES

Department of Medicine, Tulane University School of Medicine, New Orleans and the Louisiana State Health Department

Although much information has been available on the immune response and protective effects resulting from influenza vaccines, the novelty of the Asian strain of influenza A provided an opportunity for pre-epidemic investigations in populations that were essentially

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free of antibody(1). Such data were desirable in order to evaluate the immunogenicity of a new antigen and its behavior in the inexperienced host(2). Variations in antibody titers dependent upon amount of vaccine and route of administration, upon methods employed for determination of antibody, including preparation of the antigen used for this purpose, are here described.

Materials and methods. Strains of Asian virus used, technics for determination of hemagglutination-inhibition (HaI) and neutralizing antibody and methods of preparation and maintenance of monkey kidney tissue cultures (TC) have been described(3). Monovalent vaccines for experimental use, obtained from commercial sources, had been prepared from Asian virus grown in the allantoic sac of embryonated eggs and purified and concentrated by adsorption-elution with fowl erythrocytes or by protamine precipitation followed by centrifugation. Determinations of chick cell agglutinating (CCA) units were made in a Klett photoelectric colorimeter according to procedures described by Hirst as modified by Stanley(4,5). Equivalent results that were also reproducible were obtained with a Beckman, Jr. spectrophotometer ($\lambda = 545$). Titers that were greater, but that also showed the same relative differences between various lots of vaccine were also obtained by replicate determinations by the hemagglutination pattern technic with pooled fowl erythrocytes. An October 1957 lot of vaccine containing 400 CCA units/ml and previously checked with standard vaccine from the Biological Control Division, Nat. Inst. of Health was accepted as a baseline. Adjustment of CCA unit determinations was made accordingly in order that results would be equivalent to those of a standardized vaccine. Individuals, 20 to 35 years of age, were vaccinated during the period from July to Oct. 1957. Excluded from analysis of results were all persons who developed a clinical illness resembling influenza or all those who had pre-vaccination hemagglutination-inhibition antibody titers greater than 1-8.

Effect of route of administration on antibody response. Comparison of the effect of intradermal, subcutaneous or intramuscular administration of various amounts of vaccine can be made from the titers of HaI antibody that resulted (Table I). Although differences ascribable to route of administration were not produced with small amounts of antigen, a slight advantage of the intradermal route was shown following inoculation of larger quantities of vaccine. This did not appear sufficient to compensate for the higher antibody titers obtained after the larger doses that were feasible by the intramuscular or subcutaneous routes.

Effect of dosage and spacing of inoculations. In general the total amount of vaccine in terms of CCA units injected determined the titer of antibody that resulted. Whether the inoculation was given as a single injection or divided into 2 doses did not appear to affect the final outcome. Possibly, an interval of more than 3 weeks would have allowed a recall response. Nevertheless, there were fewer and milder reactions when the vaccine was given in divided doses, and this was a consideration when 400 CCA unit material was used.

Evaluation of hemagglutination-inhibition antibody titers after vaccination. To compare the magnitude of the post-vaccinal antibody response to that following natural infection the geometric mean hemagglutination-inhibition titer of a large group of convalescent sera was obtained as shown at the bottom of Table I. Since determinations were done with monkey kidney culture propagated Asian virus and sera were treated with receptor destroying enzyme of *Vibrio cholerae* for titrations of antibody in post-vaccinal as well as post-influenzal sera a basis of evaluation was obtained(3). Differences in titer could accordingly be used in comparing results of studies in other laboratories even when dissimilar virus strains or methods were employed. Because there were low titers of inhibition in some acute sera, results were analyzed in terms of fold increase as well as final titers. It can be seen that few of the vaccinated groups had antibody increments as great as those with natural illness. In addition to comparison of results with those ob-

TABLE I. Hemagglutination-Inhibition Antibody Titers Two Weeks after Various Dosages and Routes of Administration of Monovalent Asian Influenza Vaccine in Adult Humans.*

Vaccine	Route and dose in ml†	CCA units	No. tested	% with fold increase of 8 or more	% with titer of 8 or more	Geometric mean fold increase	Geometric mean titers
A	I.D. .1	7	38	11	24	2	3
A	S.C. .1	7	34	3	18	1	2
A	I.M. .5	35	71	13	30	2	3
C	I.D. .1	10	61	28	77	4	11
C	I.M. .2	20	12	33	83	6	15
D	I.M. .5	20	37	5	27	2	2
D	I.M. 1.0	40	54	26	48	3	6
C + C	I.D. .1	10	35	37	87	5	14
	+ I.D. .1	+ 10					
C + C	I.M. .2	20	5	20	80	4	14
	+ I.M. .2	+ 20					
D + D	I.M. .5	20	25	24	56	3	6
	+ I.M. .5	+ 20					
A + B	I.D. .1	7	16	50	88	7	14
	+ I.D. .1	+ 40					
A + B	S.C. .1	7	9	22	50	3	6
	+ I.D. .1	+ 40					
A + B	I.M. .5	35	14	64	93	8	23
	+ I.D. .1	+ 40					
A + B	I.D. .1	7	5	80	80	5	9
	+ I.M. .5	+ 200					
A + B	S.C. .1	7	8	63	88	8	16
	+ I.M. .5	+ 200					
A + B	I.M. .5	35	13	69	92	11	22
	+ I.M. .5	+ 200					
A + B	I.M. .5	35	11	100	100	16	41
	+ I.M. 1.0	+ 400					
Natural infection			177	73	100	11	29

* HaI antibody titers are expressed in terms of initial dilution of serum. Monkey kidney propagated Asian strain influenza A was used in all cases. Serum was treated with RDE.

† Injections were separated by an interval of 3 wk. Convalescent sera were obtained 2 wk after each inj. I.D.—intradermal, S.C.—subcut., I.M.—intramusc.

tained from influenza cases, consideration of the passage history of the virus used appeared relevant to interpretation. As described in an accompanying report(3), antibody titers in convalescent sera from natural illness determined with the TC line of Asian virus were greater than those obtained with the egg (E) or egg- ferret- mouse-egg (EFME) line. In post-vaccinal sera, however, titers with the EFME line were equivalent to those with TC virus (Table II). Accordingly, if one chose the mean antibody response produced by natural illness as the desirable goal for satisfactory response to vaccination, it would be preferable to utilize results obtained by methods that yielded maximum titers. On the other hand, employment of the E virus line to determine mean antibody titer of a group of convalescent sera would make it necessary to obtain antibody response to vaccine 2- to 4-fold greater than this mean when both are determined with the same E line(6).

TABLE II. Mean Hemagglutination-Inhibition Antibody Titers Determined with TC and E Lines of Asian Virus.*

	Virus			
	TC_{2-4}	E_1	E_7	EFME
Treatment of serum	RDE	△56°C	RDE	Periodate
Pre-vaccination serum titer	<4	<4	<4	<4
Post-vaccination serum titer	50	13	17	50

* Geometric mean titers determined from 14 paired sera.

Comparison of HaI and neutralizing antibody titers determined with TC and E lines of

TABLE III. Mean Neutralization Titers Determined with TC and E Lines of Asian Virus.*

	Virus			
	TC_{2-4}	E_4	E_8	EFME
Host system	TC	Egg	Egg	Egg
Pre-vaccination serum titer	<4	<4	<4	<4
Post-vaccination serum titer	68	8	15	16

* 10^3 ID_{50} of virus was added to dilutions of heat inactivated sera. Results are geometric mean titers determined from 6 paired sera.

Asian virus. Variations in HaI antibody titers dependent upon the line of virus used are shown in Table II. In each case the optimum method of removal of non-specific inhibitor was used(3). In contrast to the results with post-influenzal sera, titers with the EFME line were equal to those obtained with the TC line. Although a lesser degree of reactivity of the E lines as compared to the TC line occurred with neutralizing antibody (Table III), this was not nearly as pronounced as that with post-influenzal sera. The difference was especially striking when the titers obtained with the E_8 virus in post-influenzal and post-vaccinal sera were compared. Thus, it appears that specificity of antigen or antibody as well as avidity or reactivity must all be considered in explaining variations in antibody titers.

Discussion. Although the degree of protection against infection or illness is the test of a vaccine in the final analysis, correlative data can be obtained by other means of antibody measurement. With viruses such as the influenza group natural alterations in antigenic composition necessitate a corresponding change in vaccine. Accordingly, rapid means of evaluating efficacy of vaccine prior to epidemic spread are essential. Differences in antibody titers dependent upon the passage history of the virus as described herein indicate some of the problems that might be encountered in such an endeavor. Even determinations of the amount of virus in the vaccine has limitations due to inaccuracies of the quantitative aspects as well as variations in response due to immunogenicity and antigenic specificity of the strain. The results of these studies suggest that a vaccine prepared with monkey kidney propagated virus might have produced a more specific antibody to the natural virus. Although this problem was overcome by larger egg vaccine doses, there were advantages to smaller amounts of virus such as a reduction in toxic reactions. Specificity of the antigen is probably not always as noticeable unless a major antigenic change has occurred in the virus. Thus, it was not possible to show antigenic differences between the 1953 influenza A human tissue culture and E lines by HaI with human convalescent sera from natural illnesses(7).

Summary. The route of administration, either intradermal, subcutaneous, or intramuscular, did not significantly affect the antibody response following Asian influenza vaccination. Antibody titers produced were proportional to the total amount of vaccine inoculated whether given as a single dose or divided into 2 injections separated by an interval of 3 weeks. Hemagglutination-inhibition antibody titers in post-vaccinal sera were greater when determined with the TC and EFME lines of Asian virus than with early or later passages of the straight E line. Neutralization titers with the TC line in monkey cultures were considerably greater than those determined with the E lines in the allantoic sac. The geometric mean hemagglutination-inhibition antibody titer of a large group of post-influenzal sera was used as the baseline for evaluation of antibody response to vaccine by using the same virus line and method of inactivation of non-specific inhibitor in sera in both cases.

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Animal Responses to 2-Deoxy-D-Glucose Administration. (24268)

BERNARD R. LANDAU AND HERBERT A. LUBS (Introduced by Roy Hertz)
Department of Health, Education and Welfare, P. H. S., National Cancer Institute, Endocrinology Branch, Bethesda, Md.

Wick(1) has demonstrated that following 2 deoxy-D-glucose (2-DG) administration there is a decreased requirement for glucose to maintain a constant blood glucose concentration in eviscerated-nephrectomized rabbits. 2-DG has been reported to inhibit growth of experimental animal tumors(2) and to inhibit glycolysis in leukemic cells(3), slices of Walker 256 carcinoma(4) and Krebs carcinoma ascites cells(5). These observations are consistent with the hypothesis that 2-DG is a metabolic blocking agent for glucose. A survey of the response of several animal species to 2-DG was undertaken prior to clinical studies to be reported elsewhere(6).

Materials and methods. 2-DG was obtained from the Aldrich Chemical Co., Milwaukee, Wis. Blood 2-DG concentrations were determined by the Quinaldine method(7) and blood glucose concentrations by a modification of the Nelson method(8).

a) In acute toxicity studies 2-DG in doses of 1-3 g/kg body weight as a 10-40% aqueous solution was administered(1) rapidly by tail vein to 20 Swiss mice, each weighing 20 g, and (2) by ear vein over a 2-minute period to white New Zealand rabbits weighing 1 kg. Blood glucose concentrations before and blood glucose and 2-DG concentrations after infusion were determined in the rabbits.

b) In chronic studies 20 male rats of the Sprague-Dawley strain of initial weight 50 g and on a standard purina diet, were divided into 5 groups of 4 rats each. Group I received 0.5 cc of distilled water s.c. twice daily (8 a.m. and 4 p.m.); Group II received 200 mg of 2-DG/kg initial body weight in 0.5 cc of distilled water s.c. twice daily for a total dose of 400 mg/kg; Group III received 400 mg/kg twice daily; Group IV 600 mg/kg twice daily; Group V 800 mg/kg twice daily. Body weight was checked frequently throughout the study. Administration was discontinued after 19 days, and 2 hours after the last injection 2-DG and glucose concentrations were determined on tail vein blood. One week later fasting blood glucose concentrations were determined.

c) On each of 3 white New Zealand rabbits, fasted 24 hours and weighing 3-4 kg, an intravenous glucose tolerance test (500 mg/kg) and an intravenous 2-DG tolerance test (200 mg/kg), and an oral 2-DG tolerance test (400 mg/kg administered by stomach tube) was performed. Also a glucose tolerance test and glucose-insulin tolerance test (0.5 units/kg)* was performed on each rabbit following intravenous administration of 2-DG (200 mg/kg). There was a time interval of several days between tests.

Results. Mice. The LD_{50} for mice receiving 2-DG acutely by tail vein was between 2 and 3 gm/kg *body* weight. Within 5 minutes after administration the mice became ataxic and then were unable to right themselves. Respirations became rapid and shallow and convulsions occurred occasionally. The mice then became unresponsive to painful stimuli. At a dose of 1 g/kg after about 45 minutes, recovery was noted. It was only after several hours that recovery was observed in mice receiving the higher doses.

Rats. The results of administration to rats of 2-DG subcutaneously over a 19-day period

* Glucagon-free-insulin was kindly provided by Dr. W. R. Kirtly, Eli Lilly and Co., Indianapolis, Ind.

TABLE I. Weight, Blood Glucose and Blood 2-DG Levels in Rats Administered 2-DG.

Group	Dose (mg/kg/day)	Initial wt (g)	Wt, day 19 (g)	Blood glucose day 19	Blood 2-DG day 19	Blood glucose day 26
				(mg %)		
I	0	47 ± 1*	126 ± 9	129 ± 4	0	76 ± 3
II	400	53 ± 1	145 ± 4	135 ± 5	2 ± 1	—
III	800	52 ± 1	111 ± 10	162 ± 12	—	—
IV	1200	49 ± 1	96 ± 13	190 ± 10	6 ± 1	68 ± 4
V	1600	45 ± 1	96 ± 10	212 ± 40	16 ± 3	61 ± 2

* Mean ± stand. error is given.

is presented in Table I. Weight gain was less in those rats receiving the higher doses of 2-DG than in the control rats. Blood glucose concentrations 2 hours after the last injection of 2-DG were elevated above the concentration in the control group, but one week later (last column of the table) fasting blood glucose concentrations were similar in the control group and the 2 groups that received the highest doses of 2-DG.

Rabbits. Within a few minutes after intravenous administration of 1-3 g of 2-DG to a rabbit there was marked incoordination of the extremities. Then the limbs became limp; there was dorsiflexion of the neck; pupils became dilated; occasionally the rabbit cried out. Nystagmus was observed in one case. Breathing was gasping in nature. Death occurred within 6-32 minutes after completion of 2-DG administration. Table II presents the pre- and post-sugar concentrations of these animals and their survival time.

Fig. 1 presents graphically the blood sugar tolerance test responses of 1 of 3 rabbits. All had similar responses. An intravenous glucose tolerance test was performed and several days later an intravenous 2-DG tolerance test (Fig. 1-A). Following administration of 200 mg/kg of 2-DG intravenously, there was a rise in blood glucose to about twice the fasting level. Blood glucose concentrations were high for at least 5 hours with the peak concentration occurring between 1 and 2 hours after administration. In a glucose tolerance test performed 40-50 minutes after 2-DG administration blood glucose decreased much more slowly than on the control tolerance test (Fig. 1-B). A glucose tolerance test with simultaneous administration of insulin (0.5 units/kg) after 2-DG administration resulted in a response similar to the control glucose tolerance test (Fig. 1-C). Blood 2-DG concentration 5-10 minutes after 2-DG administration was near 50 mg%, but decreased rapidly. No effect of insulin or glucose infusion on 2-DG blood levels was noted. 2-DG in doses of 400 mg/kg given by stomach tube (Fig. 1-D) resulted in blood glucose level rises to about 1½ times fasting levels with the peak concentration at about 2 hours and elevated levels for at least 5 hours.

Discussion. Acute administration of 2-DG. The train of symptoms observed in mice and rabbits following intravenous administration of large doses of 2-deoxyglucose is similar to those reported following insulin administration. Woodward and Hudson(9) have demonstrated marked inhibition of glycolysis of brain slices in the presence of 2-DG and 2-DG does enter into the cerebrospinal fluid

TABLE II. Blood Glucose and Blood 2-DG Levels on Intravenous Administration of 2-DG to Rabbits.

Rabbit	Dose (g/kg)	Control blood glucose	Post-2-DG blood glucose	Post-2-DG blood 2-DG	Time of blood sample (min.)*	Time of death (min.)*
		(mg %)				
1	1	111	177	247	18	Lived
2	1	95	183	146	21	26
3	2	113	171	355	6	6
4	3	—	196	441	12	15
5	3	—	175	568	32	32

* Time is given in min. post-2-DG administration.

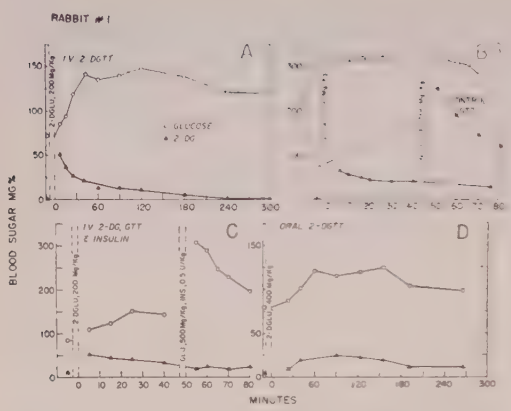

FIG. 1. Rabbit blood glucose and 2-DG tolerance tests: (A) intrav. 2-DG tolerance test; (B) control intrav. GTT and a GTT after 2-DG administration; (C) GTT with insulin following 2-DG administration; (D) oral 2-DGTT.

rapidly after intravenous administration of 2-DG to dogs(10). Sols and Crane(11) have demonstrated that 2-DG is phosphorylated by brain hexokinase but is probably metabolized no further. It therefore appears possible that the symptoms are a result of inhibition of glucose entrance and/or utilization by the brain in the presence of 2-DG, in effect, a "cellular hypoglycemia."

The symptoms of acute administration of 2-DG are very similar to those described by Herring and Hynd(12) on administration of glucosone to mice and rabbits. They noted the similarity of the symptoms to those seen after insulin administration. These authors believed that glucosone might have produced a hypoglycemia for although the reducing power of the blood was high, no methods were available for distinguishing reduction due to glucosone injected from glucose actually present. Glucosone is a hexokinase inhibitor and it seems probable that the symptoms observed were related to an inhibition of glucose phosphorylation by the brain(13).

It is also interesting to note that Winter (14), while searching for glucose analogs that would result in recovery of animals in insulin induced convulsions, administered 2-DG to rabbits. He noted a rise in blood sugar and the presence of sugar in the urine, but felt that it was inconceivable that the sugar he was measuring could be glucose since the rabbits remained in convulsions.

Chronic administration of 2-DG. Weight loss on chronic administration of 2-DG has been reported by others(15). Whether this is the result of a diabetic-like state or decreased food intake has not been determined. While the rats receiving 2-DG did have an elevated blood sugar, the concentrations returned to control values following cessation of 2-DG administration.

Tolerance tests. 2-DG enters the blood following oral administration to rabbits and is apparently associated with elevation of blood glucose concentration under our experimental conditions. Sokoloff(15) has reported moderate hypoglycemia in rats receiving 2-DG in their diet. Sols(16) has demonstrated passage of 2-DG across the guinea pig intestinal mucosa and the failure of 2-DG to inhibit glucose transport. The elevation of blood glucose concentration on intravenous administration of 2-DG to the intact rabbit is in agreement with Wick's(1) observations in the eviscerate rabbit. The glucose tolerance test following 2-DG administration is difficult to interpret since the glucose concentrations represent the glucose tolerance curve superimposed on a rising glucose concentration secondary to the 2-DG already administered. However, the tolerance tests have been done when the glucose levels following 2-DG administration are near the peak values. It thus appears that glucose disappearance from the blood is significantly prolonged by 2-DG and that in insulinized animals this prolongation is less. This complements Wick's observation that the amount of glucose oxidized by insulinized eviscerated rabbits receiving 2-DG is about the same as that oxidized by eviscerated animals not receiving insulin or 2-DG.

Summary. The response of mice, rats and rabbits to acute and chronic administration of 2-deoxyglucose (2-DG) has been studied. 1. Mice and rabbits were given large doses of 2-DG intravenously and had symptoms similar to those during insulin hypoglycemia. It is suggested that the symptoms were the result of a cellular glucopenia secondary to inhibition by 2-DG of glucose utilization. 2. Rats receiving 2-DG chronically by subcu-

taneous administration had elevated blood glucose concentrations and weight loss. On discontinuation of 2-DG administration blood glucose concentrations returned to control values. 3. Rabbits following intravenous or oral administration of small doses of 2-DG had a rapid rise in blood glucose concentrations. The glucose concentrations remained elevated for at least 5 hours. In glucose tolerance tests done shortly after 2-DG administration glucose concentrations decreased more slowly than in control tolerance tests, but the animals did respond to insulin administrations.

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Two Local Hemorrhagic Skin Responses to Bacterial Endotoxin in an Inbred Mouse Strain. (24269)

WILLIAM F. ARNDT AND HOWARD A. SCHNEIDER
Rockefeller Institute for Medical Research, New York City

Although the localized Shwartzman reaction was long undetected in mice and rats, Homma(1), and more recently Kelly *et al.* (2), have demonstrated that this phenomenon, or an analagous one, does indeed occur with adequate "preparation" or in properly selected mouse strains. In addition to the normal Shwartzman reaction, the latter workers have described a "Shwartzman-like reaction" which differs from the classical phenomenon in that it is produced by but a single injection of bacterial polysaccharide. In other respects, however, this reaction is indistinguishable from the normal 2-injection phenomenon.

It was the purpose of the experiments reported here to confirm and extend the abovementioned observations in an effort to elucidate the mechanisms underlying the single-injection reaction, which, because of its morphological characteristics, was thought to be related to the local Shwartzman reaction.

The following experiments were performed with a mouse strain bearing a genetic relationship to one of the "positive" strains (Rockefeller "pen-bred") described by Kelly *et al.*, but differing in that it now reflects some 44 generations of brother-sister inbreeding. Such a strain offers the advantage of genetic homogeneity, thereby defining an important variable of this system.

Materials and methods. The mice used were from a recently established pen-bred subline of the brother-sister, 44 generations inbred BSVS strain, originally extracted by Webster(3) from the Rockefeller Institute Stock. They are maintained under conditions of constant temperature (80° F.) and relative humidity (50%) with a uniform 12 hour artificial (fluorescent lamp) light day.

TABLE I. Skin Reactions in BSVS Mice following Injection with *E. coli* Endotoxin.*

Exp. No.	No. & sex		No. mice with skin hemorrhage I.D. only R/T†	% reactors	I.D. & I.P. R/T†	% reactors
1	12	♂	12/12	100	—	—
	18	♀	18/18	100	—	—
2	10	♂	1/10	10	9/9	100
	10	♀	2/10	20	8/8	100
3	20	♂	20/20	100	—	—
	8	♀	8/8	100	—	—
4	10	♂	8/10	80	1/2	50
5	10	♀	0/10	0	9/10	90
6	13	♂	13/13	100	—	—
	13	♀	13/13	100	—	—
Totals	124		95/124	76.6	27/29	93.1

* Prep. No. 0127 A, B from Difco Labs., Detroit, Mich.
† R/T = reactors/total No. tested.

The standard diet was Purina Laboratory Chow and tap water supplied *ad lib.*

Except where indicated, the bacterial endotoxin employed was commercially available (Difco) *E. coli* purified lipopolysaccharide (Lots No. 0127 A, and B), which, as tested by us according to the method of Reed and Muench(4), has an LD_{50} of 150 μg for 20 g adult BSVS mice when injected intraperitoneally.

For intradermal (I.D.) and intraperitoneal (I.P.) injections, the endotoxins were dissolved in normal saline. The standard volume for all I.D. injections was 0.05 ml delivered through a No. 27 needle. The volume for all I.P. injections was 0.5 ml.

Experimental results. In a series of experiments with these mice, confirming Kelly's observations, we have observed both the normal Shwartzman reaction and the single-injection "Shwartzman-like reaction." The latter occurred after as little as 25 μg of *E. coli* endotoxin or 50 μg of *S. marcescens* endotoxin* was injected into the skin, appearing after 18 hours, and reaching maximum intensity in from 24-48 hours. The intensity of this reaction was apparently dependent, to a degree, on the dosage since in some mice a slight difference could be noted between the responses to 30 μg and 50 μg of the *E. coli* material. This difference was not marked however, and the 30 μg dose was chosen as a standard for these experiments.

Maximum lesions produced averaged 15 x 20 mm, and were composed of an intense area of hemorrhagic necrosis surrounded by a margin of erythema. Lesser lesions varied from scattered petechiae to that just described with all gradations between.

The results of 6 separate experiments are summarized in Table I. In these experiments, the mice received 30 μg of endotoxin I.D. All animals were scored for number and intensity of reactions after 24 hours. At this time, those mice showing no reactions at injection site were challenged I.P. with 30 μg of the same material. Twenty-four hours following the second injection, all previously negative skin sites were again scored for hemorrhagic necrosis. Table I shows that 93% of the mice not responding to a single injection of endotoxin exhibited lesions when challenged I.P. after 24 hours. The other fact clearly demonstrated was that response to a single injection was quite variable among experiments, ranging from 0% to 100%. In view of the uniform response of these mice to 2 injections, such marked variability to the single injection in a system of presumed genetic homogeneity presented an interesting problem. Our attention was next focused on those environmental variables which might explain these events. A previous history of transient bacterial epizootics in the colony, coupled with the fact that the inciting substance for these lesions was a bacterial product, led to the hypothesis

* Lot P-25, obtained from Dr. M. Shear, Nat. Inst. of Health, Bethesda, Md.

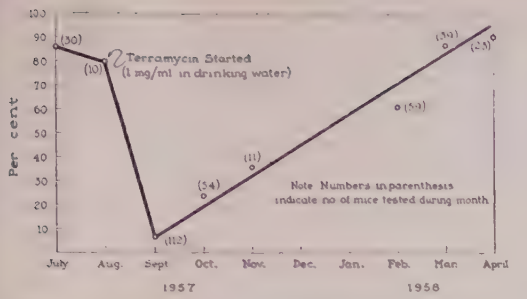

FIG. 1. Frequency of hemorrhagic skin response of BSVS mice to single intradermal inj. of *E. coli* endotoxin.

that the "natural" flora in the animals were related, in some way, to the observed variability. To test this proposal, a large portion of the colony was placed on oral oxytetracycline therapy by dissolving the antibiotic (Pfizer Animal Terramycin) in drinking water in a concentration of 1 mg active drug per ml. The effect of this therapy over a course of 8 months is shown in Fig. 1. This curve represents the percentage of mice that gave a positive reaction when tested with a single 30 μg I.D. injection of endotoxin. Each point is the average percent response of all experiments conducted during that month. Therapy was begun where indicated and continued, at the same level, throughout the following months. In Sept. 1957, the period during which the greatest effect was seen, a corresponding control group of 57 mice reared on tap water showed an average reactivity of 65% as opposed to the 7.1% observed for the experimental group. A chi-square test for the difference between these groups showed a $P<<0.001$. A similar test for random deviation from the mean of all the experimental groups during the course of the therapy showed χ^2 to be 142 ($P<<0.001$).

Preliminary results indicate that chloramphenicol is also effective in this regard, although its use has not been sufficiently long to permit the rise of any resistance effect such as was noted with Terramycin.

Discussion. The preceding data obtained with BSVS mice confirm the observations of Kelly *et al.* on the related Rockefeller strain. In contrast with the latter report however, BSVS mice have consistently displayed a higher frequency in response (93% in BSVS vs. 57% in Rockefeller "pen-bred") to 2 injections (I.D. + I.P.) of endotoxin. Further, in BSVS mice, doses of endotoxin one-tenth that employed by Kelly and co-workers were sufficient to incite lesions in the single-injection system. From these findings, it would seem that the role of the host genotype is important not only in a qualitative sense, but in a quantitative way as well.

Perhaps the most interesting aspect of these observations was the single injection phenomenon. It would appear that this reaction is a special form of the Shwartzman reaction inasmuch as both are so similar in their gross histological characteristics. It seems clear that Terramycin had an effect on the responsiveness of the mice in this regard, but that this effect diminished on continued therapy. Such data suggest the role of a microbial agent, initially sensitive to the antibiotic, but gradually developing some resistance on continuing exposure. Investigations currently in progress deal with the problems of isolating the microorganisms presumably responsible for increased "sensitivity" of these mice toward bacterial endotoxin.

Summary. The observations previously made by Kelly *et al.* on production of hemorrhagic skin lesions by dermal injection of bacterial endotoxin in Rockefeller mice have been repeated with the genetically related, highly inbred, BSVS strain. BSVS mice displayed all of the reactions reported by the above authors, but with a significantly increased frequency, and at much lower doses of endotoxins. Pretreatment with Terramycin caused a marked decrease in frequency of reactors to a single injection of endotoxin. This effect gradually diminished over a period of 8 months of continued treatment. It is suggested that the single-injection reaction is a special form of the dermal Shwartzman reaction in which some microbial agent(s) plays an as yet unknown part.

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Amylase in Fallopian Tubes.* (24270)

Robert L. McGeachin, Lila A. Hargan, Betty Ann Potter and Arthur T. Daus, Jr.
Department of Biochemistry, University of Louisville School of Medicine, Louisville, Ky.

We have been interested in locating the site of synthesis of extrapancreatic, extrasalivary amylase(2) in animals. Green(1) has recently shown that fluids from various cysts associated with human Fallopian tubes have amylase contents of from 1,100 to 80,000 Somogyi units per 100 ml, or 10-700 times the normal human serum amylase level. Since these cysts had epithelial linings which were morphologically identical to the epithelium lining of the Fallopian tubes, Green postulated that this epithelium secretes an enzyme whose physiological substrate is the intraluminal glycogen produced by secretory endometrium. His determinations indicated that the enzyme is an alpha-amylase. However, since his work dealt only with the cystic fluids and not the Fallopian tubes, we were interested in determining the amylase contents of the tubes themselves. This report deals with such observations in various species, including man.

Methods and materials. Amylase activities of both sera and Fallopian tubes were determined by Van Loon's amyloclastic method (3). All values reported are in Van Loon units, numerically identical to Somogyi units, per 100 ml of serum or 100 g of tissue. The Fallopian tubes and sera were obtained from stock animals in the case of the rat, dog, guinea pig and cat, from animals at the abbatoir for the cow, hog and sheep. All of the animals used were adult, sexually mature females with the exception of one group of monkeys as noted in the table. Human specimens were provided by the Dept. of Obstetrics and Gynecology and Pathology, Univ. of Louisville School of Medicine. The Fallopian tubes were carefully freed of excess surrounding tissue and kept frozen at –18°C until used for analysis. Fluids for analysis were obtained by homogenizing the tubes with 0.9% NaCl, first in a Waring blendor and then in a Dounce glass homogenizer(4). The final dilutions with 0.9% NaCl were 1:10 to 1:50, varying with the animal used.

In some of the human specimens small parovarian or paroophoron cysts were found in the tubes or in the tissue surrounding the tubes. These cysts were carefully removed and their fluids also analyzed for amylase.

In some of the experiments on dogs and rabbits, one tube was removed and a blood specimen taken, then pilocarpine was injected and after 30-45 minutes the other tube was removed and another blood specimen taken.

Results. Our data on the fluids from human Fallopian tube cysts (Table I) show amylase levels many times that of human serum, in agreement with Green's findings. The amylase levels of the Fallopian tubes themselves are much lower, although still several times higher than serum amylase. For other animals, the Fallopian tube amylase levels (Table II) were greater than serum amylase levels only in the rabbit, the cow and the sheep. In the rat, dog, hog, guinea pig, cat and monkey, the serum amylase levels were higher than the corresponding tubal amylase levels. The amylase levels of tubal secretions were not appreciably increased in the dog and rabbit by injection of pilocarpine, al-

TABLE I. Amylase in Human Fallopian Tubes and Fallopian Tube Cysts.

Serum amylase	Fallopian tube amylase	Cyst fluid amylase	Type of cyst
—	410	—	—
—	400	5,010	Parovarian
88	147	—	—
50	565	—	—
—	313	29,100	Paroophoron
110	391	37,100	"
	309	1,940	Parovarian
	132	—	—
	822	14,500	Paroophoron
	265	—	—
—	76	—	—
—	85	—	—

* This work was supported by a research grant from Nat. Inst. Health.

TABLE II. Amylase of Fallopian Tubes of Various Species.

Animal	No. of animals	Fallopian tube amylase		Avg serum amylase
		Range	Avg	
Rat	3	477– 687	575	2250
Dog	4	357–1125	500	770
Monkey (mature)	6	30– 640	215	585
Monkey (immature)	6	250– 278	264	685
Rabbit	5	223– 614	371	236
Guinea pig	4	98– 440	201	2070
Cat	2	175– 220	200	1020
Cow	6	27– 254	170	79
Sheep	1	330	—	110
Hog	3	485– 700	590	2025

though serum levels in the dog were increased.

Since Green indicated that amylase was not found in the cysts from preadolescent girls, tubes and sera from both mature and immature monkeys were analyzed. There was no noticeable difference between these two groups.

Discussion. It is evident that, except in the human cysts, amylase is not found in the Fallopian tubes in quantities comparable with the pancreas and salivary glands(2). Since the Fallopian tubes do not have any secreting glands but only a secreting mucosa which makes up a relatively small proportion of the total organ, perhaps one should not expect a very high amylase level even if amylase is synthesized in the tubes. However, in the human, cow, rabbit and sheep, where the Fallopian tube amylase levels are greater than the serum levels, at least some of that amylase must be synthesized by the Fallopian tubes. The only other possible explanation would be collection and concentration of amylase from the serum by the tubes.

In the other animals tested, the fact that serum amylase levels are higher than tubal amylase allows one to say only that the amylase in the tubes may have been synthesized therein. Where the serum amylase levels are higher than Fallopian tube amylase levels, it is possible that the amylase found in the tubes *could* have come from the serum since amylase seems able to penetrate most body tissues(2).

It should be pointed out that the serum amylase levels of monkeys, not previously reported in the literature, ranged from 314 to 860 Van Loon units per 100 ml in 12 animals, with a mean value of 635. This is much higher than the normal human serum levels of 60-180.

Summary. Amylase levels in the Fallopian tubes of the human, cow, rabbit and sheep are greater than the corresponding serum levels, hence amylase must be produced in the tubes of these animals. Amylase is also found in the Fallopian tubes of the rat, dog, hog, guinea pig, cat and monkey but in lower concentrations than the corresponding sera. High levels of amylase are found in the fluids from cysts associated with human Fallopian tubes.

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Serum Glycoproteins in Monozygotic and Dizygotic Twins.* (24271)

W. LeRoy Heinrichs and M. R. Shetlar
Department of Biochemistry, University of Oklahoma School of Medicine, Oklahoma City, Okla.

Numerous studies have been reported concerning alterations of serum glycoproteins in pathological states. The field has recently been reviewed by Stary(1) and by Winzler (2). However, few studies(3,4,5) have dealt with the variations found in normal individuals and the factors which influence them. This study was undertaken to evaluate the effect of genetic factors on the serum glycoprotein levels.

Methods. Subjects were volunteer twins from the Oklahoma City schools and ranged from 13 to 19 years of age. A medical history was taken, fingerprints were made, and anthropometric data was recorded at the time that blood samples were taken. The twins were divided into monozygotic and dizygotic groups according to the criteria of Newman (6).

Paper strip electrophoresis studies of protein and glycoprotein were made as previously described(7). Total serum glycoprotein was quantitated by hexose determination by the tryptophan method(8). Seromucoid was isolated by the method of Weimer and Redlich-Moshin(9) and quantitated by the tryptophan method. Total serum protein was determined by the biuret reaction.

Results. Total glycoprotein hexose averaged 122 mg per 100 ml (range 98-142); seromucoid averaged 12.5 mg per 100 ml (range 10-18) for the total group. No significant difference in regard to these results was found between the monozygotic and dizygotic groups, or between either group of twins and control group of normal adults. The statistical studies of the differences between monozygotic pairs as contrasted to the differences between dizygotic pairs are summarized in Table I. In nearly all cases average differences between monozygotic pairs were found to be smaller than average differences between dizygotic pairs. However, only total glycoprotein hexose and albumin protein were significantly lower at the 1% level of significance.

Since a trend toward closer agreement between identical twins as compared to fraternal twins occurs, it would appear that the concentrations of the various serum protein and glycoprotein fractions are controlled partly by genetic factors. The genetic factors, however, would appear to be of minor importance in the normal individual at adolescence. Since inflammatory conditions, even those minor in nature, are known to have noticeable influence on the serum glycoprotein level, it may be postulated that the recent history of the individual may have more effect than the genetic factors. This postulation is borne out by the data from one pair of monozygotic twins; one twin, who had rheumatic fever 4

TABLE I. Comparison of Monozygotic and Dizygotic Twin Pairs.

Constituent	Avg difference between twins*		
	Monozygotic	Dizygotic	T value
T. protein	.22	.41	1.66
Glycoprotein hexose (mg/100 ml)	5.5	16.6	2.87†
(Glycoprotein + T. protein) × 100	.06	.16	2.18‡
Seromucoid	.8	1.5	1.20
Protein fractions			
Albumin	1.8	3.8	2.82†
α_1-globulin	.6	.4	.17
α_2- "	1.1	1.6	.38
β- "	1.1	1.3	.12
γ- "	1.8	3.2	1.94
Glycoprotein fractions			
Albumin	2.4	2.2	.30
α_1-globulin	1.7	2.4	1.10
α_2- "	2.9	4.3	1.12
β- "	1.8	2.5	.83
γ- "	2.4	2.7	.25

* Based on 13 pairs of monozygotic and 9 pairs of dizygotic twins.
† Significant at 1% level.
‡ " " 5% " .

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years previous to the study, had both a higher glycoprotein (136 v. 125 mg/100 ml) and a higher serum protein (7.74 v. 7.16). A second monozygotic pair included one twin with a history of a cold one week previous to sampling (glycoprotein 133 mg/100 ml v. 122 for the other twin).

Summary and conclusions. The influence of genetic factors on the levels of total serum glycoprotein hexose, seromucoid hexose, and the bound hexose of electrophoretic fractions were investigated by comparing the differences found between monozygotic twin pairs as contrasted to the differences between dizygotic twin pairs. The differences between total serum glycoprotein hexose of the monozygotic twin pairs were significantly lower than those found for the dizygotic twins. The quantity of hexose bound to various electrophoretic fractions was found to be closer between monozygotic than between dizygotic twins. However, these differences were not significant statistically. It may be concluded that genetic factors play a minor role in establishment of normal levels of serum protein and glycoprotein.

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Studies on Role of Liver in Nitrogen Mustard Detoxication.* (24272)

EBERHARD G. TRAMS (Introduced by Paul K. Smith)
The George Washington University School of Medicine and Cancer Clinic, Washington, D. C.

Skipper *et al.*, who investigated the distribution of methyl-C^{14}-bis (beta-chloroethyl) amine, (HN2), in mice observed that appreciable amounts of radiocarbon were excreted in the form of carbon dioxide(1). In our laboratory similar results were obtained in rats. It was shown that only small amounts of the C^{14}-methyl group of HN2 were transferred by transmethylation mechanisms(2). Subsequent studies showed that in the microsomal fraction of rabbit and rat liver homogenates there existed an enzyme system which could readily demethylate and oxidize the labile methyl group of HN2. It also was demonstrated that such an *in vitro* preparation removed the N-ethyl group of HN2, although at a slower rate than the methyl group(3). Thus, there was a possibility that the liver, through demethylation and de-ethylation, was able to metabolize sufficient amounts of the injected drug in a short time to justify the assumption that the observed de-alkylations represented a detoxication mechanism.

This investigation was undertaken with the aim of determining how much radioisotope was excreted by the liver *via* the lymphatic and biliary systems after intravenous administration, and if these systems still contained significant amounts of cytotoxic materials. Additional studies were carried out in order to determine if preferential routing of the HN2 through the liver influenced the toxicity of the drug.

Methods. The methyl-C^{14} bis (beta-chloro-

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ethyl) amine HCl was obtained from the U.S. Atomic Energy Commission and had a specific activity of 15.2 μc per mg. Radioactivity was assayed in a continuous gas flow proportional counter on infinitely thin plated samples. All animals used in this study were adult Osborn-Mendel rats. Collection of lymph or bile was accomplished under sterile conditions through cannulae in the thoracic duct and/or the common bile duct. The animals were placed in restraining cages and the fluids collected from PE 10 polyethylene tubing while the animals were fed and watered *ad libitum*. Non-labeled nitrogen mustard ("mustargen-Merck") was injected under ether anesthesia at concentrations of 1.0 mg/ml through the femoral vein or, following laparotomy, through the vena cava or the portal vein of the rat.

Toxicity of bile or lymph obtained from the rats was estimated by tissue culture assay. Fragments of embryonic C3H mouse hearts were grown in roller tubes. The nutrient (Gey's) was withdrawn after growth was established and exchanged for nutrient-bile or nutrient-lymph mixtures. Toxicity of bile or lymph was determined on fragments cultured for 48 hours. The tissue cultures were exposed to lymph or bile for 6 hours. Following restitution of the normal nutrient the individual fragments were graded 18 hours later on the basis of cellular rounding, granulation, and the extent of disintegration and compared with control tubes. At least 20 tissue culture fragments were employed for each determination of cytotoxicity.

Results. About 30% of the intravenously administered radioactivity from C^{14}-methyl labeled HN2 was excreted in 24 hours through the common bile duct. During the same period 15% of the radioactivity was collected in thoracic duct lymph.

Comparison of specific activity of bile, lymph and blood indicated that bile contained 3 to 4 times the radioactivity of the lymph and from 25 to 35 times that of blood at comparable intervals after injection of the radioactive drug. Assay of bile obtained at varying intervals after injection of HN2 into the rat showed that this bile did not contain sufficient cytotoxic material (or unchanged drug) to account for the radioactivity observed in these samples (Table I). Subsequent chromatographic studies indicated that there was little or no unchanged drug present in the bile. Exposure of tissue culture fragments to diluted samples of thoracic duct lymph showed that lymph contained some residual cytotoxic material (Table I). Calculations based on amount of radioactivity in samples withdrawn 2 and 4 hours after injection indicated that one-tenth of the radioactivity may have been present in the form of cytotoxic material or as unchanged HN2. Lymph and bile obtained from untreated rats failed to protect tissue culture fragments against the deleterious effects of added HN2. To determine if passage of HN2 through the liver prior to reaching the systemic circulation had an effect on overall toxicity of HN2, the drug was injected at an LD_{50} level *via* the portal vein. Control animals received the HN2 by injection into the vena cava or femoral vein. There was no significant difference between survival rates of the portal vein injected and femoral vein injected rats (Table II). The reason for the

TABLE I. Effect of Bile and Lymph from HN2 Treated Rats on Embryonic Mouse Heart Tissue Cultures.

Substance tested	Conc. of HN2 in mg/ml	% of clonal fibroblasts showing: Rounding	Granulation	% of clones showing lysis and disintegration
Bile	5×10^{-4} *	20-25	20-25	10-20
"	1×10^{-3} *	75-95	75-95	75-95
"	1×10^{-2} †	0- 5	10-20	0- 5
"	4×10^{-2} †	0- 5	25-50	0- 5
Lymph	1×10^{-3} *	75-95	75-95	75-95
"	1×10^{-3} †	0- 5	50-75	0- 5
"	4×10^{-3} †	50-75	75-95	50-75

* Known amounts of HN2 added as Mustargen.
† Amounts of HN2 calculated on basis of C^{14} content.

TABLE II. Effect of Route of Administration of an LD_{50} Dose of HN2 on Mortality Rate of Adult Rats.

Route of admin.	No. rats	No. rats died within 10 days	No. rats survived
Femoral vein	20	12	8
Vena cava	17	14	3
Portal vein	20	13	7

slightly higher mortality rate in vena cava injected animals was not evident.

Summary and conclusion. It was concluded that a major portion of intravenously injected HN2 passes through the liver of the animal. There, metabolic alterations, possibly other than simple de-alkylations, take place which lead to the biliary or lymphatic excretion of compounds the toxicity of which is greatly attenuated. It was demonstrated, however, that a single passage of the drug through the liver does not suffice to influence mortality rate due to an LD_{50} dose significantly. Detoxication of HN2 or its immediate metabolites in the liver, does not proceed at a sufficiently rapid rate to change the clinical course of mustard intoxication. This substantiates our previous observations which indicated that the effects of the drug on the organism are initiated quite rapidly following its administration.

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17-Ketosteroids in Gastric Juice.* (24273)

EDITH B. FARNSWORTH
Passavant Memorial Hospital, Chicago, Ill.

The finding of 17-ketosteroids (17-KS) in gastric juice(1) warrants a more extended investigation. The present report comprises analyses of 28 samples obtained from 16 patients who were accepted at random and without consideration of age, sex or diagnosis.

Methods. Samples of gastric juice were obtained in 2 ways: 1) aspiration of the stomach one hour after instillation of 200 cc of 10% glucose in water or, 2) aspiration by Levine tube attached to the Wangensteen suction apparatus. Those obtained by simple aspiration were taken in the morning from an otherwise fasted stomach; material removed from the suction system was taken at undetermined times. After determination of the pH and adjustment to 1.0 or slightly less, the samples were boiled for 10 minutes under a reflux condenser, cooled and extracted with ether. Separation with the Girard T reagent was practiced and the extract was again evaporated to dryness. The resultant dry material was dissolved in 5 cc of freshly distilled ethyl alcohol. After development of color with dinitrobenzene and KOH, the aliquots were read in the Coleman spectrophotometer. A standard curve was developed with each lot of unknowns.

Results. The results are detailed in the accompanying table. Values have been calculated on the basis of milligrams per liter of gastric juice.

Discussion. Comparison of the pH values with those of the 17-KS fails to suggest a direct correlation. Of greater interest is the magnitude of some of the quoted values in comparison with the negligible concentrations of 17-KS found in blood plasma. In this connection, Samuels *et al.*(2) reported that there are no measurable amounts of 17-KS in peripheral human blood. Other investigators(3, 4) using modified procedures, have carried out successful analyses of plasma for dehydroepiandrosterone (DHA) and androsterone. On the basis of these individualized methods, Gardner quotes values for normal males of 40-130 μg/100 ml, with somewhat lower figures for females. Recalling that DHA could hardly be expected to be present in association with the pH commonly found in gastric juice,

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TABLE I. 17-Ketosteroids in Gastric Juice.

Case	Sex	Date (1958)	Clinical notes	pH	& color	17-Keto. (mg/l)
1	♀	5/23	Postoperative cholecystectomy and hemicolectomy	4.5	Green	*5.0
		24		1.3	"	4.8
2	♀	23	Adrenalectomy; during surgery. Hydrocortisone IV	6.3	"	9.2
		24	Postoperative—24 hr	3.4	"	.4
3	♀	6/10	Diverticulitis with obstruction	2.1	"	2.6
		10		1.7	"	3.2
		13		1.4	"	trace
4	♀	24	Small bowel obstruction, prior to surgery; on hydrocortisone IV.	1.7	"	8.2
5	♂	15	Postoperative; partial gastrectomy	6.7	Buff	0
		15		6.7	"	0
		16		6.8	Orange	trace
6	♀	15	Colectomy. Hydrocortisone, 100 mg IV.	1.7	Yellow	4.6
7	♂	18	Partial gastrectomy	6.8	Buff	2.0
8	♂	5/27	Gastric ulcer	1.7	Colorless	†5.0
9	♂	27	Cholecystitis	2.0	"	4.2
10	♂	29	No gastro-intestinal disease	2.5	"	2.5
11	♂	29	Bleeding peptic ulcer	1.4	"	6.0
12	♂	29	Pyloric ulcer	3.0	"	4.6
13	♂	29	Cirrhosis of liver	2.3	"	2.9
14	♂	6/ 6	Duodenal ulcer	1.7	"	4.0
15	♂	6	Rheumatic heart	1.8	"	1.4
16	♀	3/22	Chronic nephritis with uremia			1.0
		24	*Idem*			2.8
		28	"			2.6
		28	"			2.6
		28	"			2.0
		4/12	"			0
		12	"			0

* Aspiration by constant suction. † Simple aspiration.

nor would it have survived the acid hydrolysis to which our samples were subjected, it is probably safe to assume that the values found in gastric juice are far in excess of what might be present in the plasma and are comparable to those found in urine.

On the other hand, intimations of correlation can be found, even in these few preliminary analyses, with intravenously administered hydrocortisone. Most strikingly suggestive is the second case, in which the first specimen was taken during surgical removal of the adrenals, while 100 mg of hydrocortisone were being administered by intravenous drip. The second sample was withdrawn on the following day, at which time the replacement therapy was given in smaller doses, by the intramuscular route, and 17-KS concentration had dropped from 9.2 mg per liter to 0.4.

It will be noted that the instances in which 17-KS were absent entirely or present only in trace were 2, a male patient who had undergone partial gastrectomy, and a female who was in the pre-terminal phase of chronic glomerulonephritis with high-grade uremia. Further studies are required for even a tentative interpretation of these findings.

Conclusions. Ether-soluble material giving a positive Zimmerman reaction is present in gastric juice in varying concentrations. Evidence is lacking of a direct correlation between acidity of the juice and concentration of the steroids. Concentration of 17-KS in gastric juice is shown to increase markedly during intravenous administration of hydrocortisone; hence it appears to reflect to some extent the blood level of hydrocortisone. The inference is justified that the gastric mucosa is the site of

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Assay of "Hemopoietine" in Starved Animals; Properties of Urinary Hemopoietine. (24274)

G. HODGSON, M. PERRETA, D. YUDILEVICH, AND I. ESKUCHE
(Introduced by A. S. Gordon)
Laboratorio de Física Nuclear Pura y Aplicada, Universidad de Chile, Santiago

The effects of plasma from anemic rabbits on plasma iron turnover of normal rabbits have been described(1). A clear increase in plasma iron turnover was produced by native and boiled plasma from phenylhydrazine treated rabbits; bled rabbits' plasma produced a slighter effect and only after dialysis. Although rich in Hemopoietine, plasma from phenylhydrazine treated animals is not an economic source for abundant material necessary for chemical fractionation studies. In view therefore of our previous findings that "Hemopoietine" appears in urine(2,3), it was decided to use urine from anemic animals, as a source of "Hemopoietine" for chemical studies. Before doing this it was necessary, however, to work out a satisfactory method for Hemopoietine concentration, and also have available an adequate test for assaying the various fractions quantitatively. Use of plasma iron turnover studies in normal rabbits as a routine assay is impractical, because of large amounts of material required. Preliminary experiments with normal rats, measuring Fe^{59} uptake in erythrocytes, indicated that this was not a satisfactory bio-assay animal either. It was with great interest therefore that we read of the experiments which demonstrated that animals with depressed erythropoiesis, presumably a consequence of low endogenous hemopoietine titers, were very sensitive to extracts from plasma of anemic animals(4,5).

This communication presents results of experiments in which starved rats with depressed erythropoiesis(5), were used as assay animals to develop a method for Hemopoietine concentration, and to describe the application of this method to the study of some properties of urinary Hemopoietine.

Method donor animals. Rabbits of both sexes, average weight 2 kg, were used. Treatment of these animals has been described(1). For urine collection the rabbits were maintained in metabolic cages, and urine collected in sterile glass flasks kept in an ice-salt mixture. The urine obtained was centrifuged and then kept frozen until further treatment.

A. *Receptor rabbits.* To test the effect of Hemopoietine on Hb synthesis in starved animals, extract of boiled plasma from phenylhydrazine treated rabbits prepared according to Borsook(6), were injected intravenously (10 ml/kg), twice a day for 2 days, in fasted male rabbits (2 kg). Animals were fasted 2 days before and during injection period. Fe^{59} was injected on morning of 5th day of fasting, and plasma iron turnover and Fe^{59} incorporation into red cells studied by methods previously described(1). The results of this experiment are summarized in Table I.

B. *Receptor albino rats.* In preliminary tests for adequate method of concentration for Hemopoietine, starved male albino rats, of weights indicated in Table II, were used. These rats were fasted 2 days previous to Hemopoietine injections and for 3 days thereafter. Hemopoietine preparations were injected intravenously (tail vein) twice a day on third and fourth day of fasting. Fe^{59}, 0.2

TABLE I. Plasma Iron Turnover and Fe^{59} in Erythrocytes of: Normal Rabbits (N.R.), Starved Rabbits Injected with: Saline (S.R.S.), Normal Rabbits' Boiled Plasma (S.R.N.R.P.) and Phenylhydrazine Treated Rabbits' Boiled Plasma (S.R.Ph.R.P.).

Group	No. rabbits	Plasma iron turnover time constant, %/hr	Plasma iron, μg/ml	Plasma iron turnover, μg/hr	Erythrocytes Fe^{59}, % dose 24 hr	48 hr
N.R.	5	43.2 ± 1.6	2.93 ± .31	106 ± 5.2	49.7 ± 5.0	60.9 ± 4.0
S.R.S.	5	22 ± 3.15	2.76 ± .83	51.2 ± 4.5	12.8 ± 1.6	36.9 ± 6.9
S.R.N.R.P.	6	18 ± 3.85	3.48 ± .42	56.7 ± 6.2	15.1 ± 3.1	38.1 ± 8.1
S.R.Ph.R.P.	9	56.4 ± 1.23	1.97 ± .31	97.4 ± 8.4	48.1 ± 4.6	74 ± 4.1

μc/rat was injected on morning of 5th day of fasting, approximately 18 hr after last Hemopoietine injection. Fe^{59} was injected intravenously (femoral vein) under light ether anesthesia, as iron-ascorbate, incubated in normal rat serum. An aliquot of dose diluted to 10 ml was kept as standard. 20 hr after Fe^{59} injection, the animals were anesthetized with ether and exsanguinated by aortic puncture using a heparinized syringe. Blood radioactivity was determined in 2 ml sample using a scintillation counter. Hematocrits were done using standard technics. In a small group of starved rats, total circulating red cell volume was determined after 5 days of starving using Fe^{59} tagged red cell method. The average value obtained: 2.29 ± 0.13 ml/100 g (pre-fasting weight) was used for calculation of Fe^{59} uptake in erythrocytes.

C. *Starved grey rats.* The studies described above were continued using male grey rats obtained from stock of Instituto de Biología Juan Noe, University of Chile. It was necessary to vary the technic a little. The receptor rats were fasted for 4 days, received subcutaneous injections of Hemopoietine, twice a day on 2nd or 3rd day and again in morning of 4th day of fasting, 1 hour prior to Fe^{59} injection. Doses given are shown in Table III 24 hr after Fe^{59} injection, the animals were anesthetized with ether, exsanguinated by aortic puncture, and liver removed for radioactivity measurements. In preliminary experiments, measuring femurs and spleen as well, most valuable information could be obtained from counting only liver and blood. In some of these starved rats total red cell volume was determined by the Fe^{59} tagged red cell method, on morning of 5th day of fasting. Advantage was taken of this experiment to determine amount of blood radioactivity remaining in liver after exsanguination. The mean circulating red cell volume in these animals was 2.42 ± 0.14 ml/100 g (pre-fasting weight) and 2.13% of total blood radioactivity was present in the liver corresponding to 100 g rat (pre-fasting weight). These values were used for calculation of Fe^{59} in erythrocytes and liver.

Results. A. Effect of boiled plasma from phenylhydrazine treated rabbits on plasma iron turnover and Fe^{59} incorporation into red cells in fasted rabbits. The results summarized in Table I show that fasting lowers plasma iron turnover to about 50% of its normal value. Fe^{59} incorporation into eryth-

TABLE II. Fe^{59} in Erythrocytes of Starved Male Albino Rats. Rats of groups 1 to 3 weighed from 180-220 g; those of groups 4-9 between 250-320 g. Abbreviations: B.P., extract of boiled plasma; R., rabbit; N., normal; Ph., phenylhydrazine treated; Al. ext., alcoholic extract; Bl., bled; U., urine.

Group	No. animals	% Fe^{59} erythrocytes	Dose of material inj./rat
(1) Saline	8	4.7 ± 1.37	8 ml 9‰ NaCl sol.
(2) B.P.N.R.	6	7.4 ± .97	8 " extract from 6 ml boiled plasma
(3) B.P.Ph.R.	15	37.7 ± 3.10	*Idem*
(4) Saline	11	11.7 ± 2.05	8 ml 9‰ NaCl sol.
(5) $B.P.Ph.R._{I}$	5	42.4 ± 4.20	10 mg Biuret positive material
(6) $Al.ext.\ B.P.Ph.R._{I}$	7	43.7 ± 3.42	*Idem*
(7) $Al.ext.\ B.P.Ph.R._{II}$	6	32.0 ± 3.60	"
(8) $Al.ext.\ U.Ph.R._{II}$	6	43.6 ± 4.20	"
(9) Al. ext. U.Bl.R.	4	24.3 ± 5.85	"

TABLE III. Fe^{59} (% of Injected Dose) in Erythrocytes and Liver of Starved Rats, Average Weight 160 g, Injected with Crude Alcoholic Extract of: Normal Rabbits' Urine (C.N.R.U.), Phenylhydrazine Treated Rabbits' Urine (C.Ph.R.U.) and Alcoholic (Al.) Extracts of the Latter.

Group	No. animals	Erythrocyte Fe^{59}, %	Liver Fe^{59}, %	Erythrocyte liver ratio, E/L	Relative specific activity, %
(1) Saline	13	10 ± .79	17 ± 1.22	.59 ± .07	
(2) C.N.R.U. 10 mg	6	11.5 ± 2.48	16.7 ± 1.91	.75 ± .22	
(3) C.Ph.R.U. 10 mg	6	50.7 ± 1.83	7.2 ± 1.25	8.0 ± 1.23	100
(4) 50% Al., pH 7, Ph.R.U. 10 mg	6	32.5 ± 2.6	10.3 ± .93	3.27 ± .46	36
(5) 50% Al., pH 4, Ph.R.U. 4 mg	6	30.7 ± 1.89	8.5 ± .75	3.7 ± .39	105
(6) C.Ph.R.U. 16 mg	4	41.8 ± 2.82	5.8 ± .44	7.4 ± .96	100
(7) 50% Al., pH 4.5, C.Ph.R.U. 16 mg	6	19.7 ± 3.5	9.9 ± 1.3	2.2 ± .18	24
(8) 50%-80% Al., pH 4.5, C.Ph.R.U. 16 mg	6	60.2 ± 5.14	4.9 ± .18	13.7 ± 2.48	193*

* Relative specific activities are calculated as ratio $\frac{\triangle \text{E/L/dose x}}{\triangle \text{E/L/dose crude}}$, where $\triangle$ E/L is increase of E/L ratio; dose x, dose of purified material, and dose crude, starting material.

rocytes, especially at 24 hr, is also notably diminished. Extracts of boiled plasma from phenylhydrazine treated rabbits increase plasma iron turnover and Fe^{59} uptake in erythrocytes significantly. Extracts of boiled normal rabbit plasma do not affect these indexes.

B. *Effect of various "Hemopoietine" preparations on Fe^{59} uptake by red cells in starved albino rats.* Table II summarizes results on effect of Hemopoietine on Fe^{59} uptake by erythrocytes of starved male albino rats. Extracts of boiled plasma from phenylhydrazine treated rabbits, notably increases the percentage of Fe^{59} appearing in red cells at 20 hr. (II-3). Extract of boiled normal plasma produces a slight increase of Fe^{59} incorporation, when compared with control and experimental series run simultaneously (II-1-2). The factor responsible for the effect of extracts of boiled anemic plasma is non-dialyzable. It flocculates on treatment with saturated $(NH_4)_2SO_4$ at pH 4.5. On treatment of water extracts obtained from boiled plasma of phenylhydrazine treated rabbits with 4 vol. ethanol at pH 4.5 an abundant precipitate formed, which was easily separated from supernatant by centrifuging. The precipitate obtained was extracted with $9^0/_{00}$ NaCl solution, and the soluble fraction was tested for activity. Table II (5,6) shows results of assay of this type of crude alcoholic fraction of boiled plasma, which has the same activity as original boiled plasma extract. The dose was 10 mg Biuret reacting material/rat. All the Biuret reacting material in solution precipitated on treatment with phosphotungstic acid, not as in the case of the aqueous extract of boiled plasma, in which only a part of the Biuret reacting material came down with phosphotungstic acid. In view of the positive result of this assay, it was decided to test for activity in extracts obtained in a similar fashion from urine collected from rabbits after 3 bleedings (once a day, 15 ml/kg) and urine from phenylhydrazine treated rabbits, collected after 5 injections of phenylhydrazine (10 mg/kg). The phenylhydrazine treated donors were exsanguinated after urine collection and the alcohol extract of boiled plasma also tested for activity. Table II (7,8,9) shows clearly that the activity of extracts of urine from phenylhydrazine treated donors, is greater than that of boiled plasma of the same donors, and the latter is greater than that of the urine of bled rabbits.

The crude urinary extract was tested for presence of phenylhydrazine using a Fiegel spot test, sensitive to 0.04 μg(7) and shown to give no color reaction.

C. *Bio-assay and properties of urinary Hemopoietine.* Fig. 1 shows the results of a quantitative bio-assay, using crude alcoholic extract of urine of phenylhydrazine treated rabbits. In Fig. 1a it can be seen that Fe^{59} of erythrocytes at 24 hr is proportional

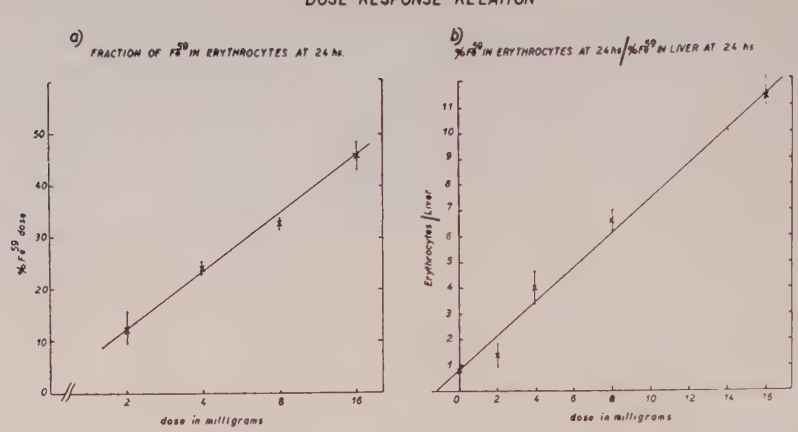

FIG. 1. Starved male rats inj. with different doses of Biuret reacting material of urine of phenylhydrazine treated rabbits.

($r > .994$) to $\log_2$ of dose of urinary Hemopoietine, expressed as mg Biuret reacting material. Fig. 1b) shows that the ratio Fe^{59} of erythrocytes/Fe^{59} of liver at 24 hr is proportional to dose of urinary Hemopoietine ($r > .998$). Table III shows results of other experiments with Hemopoietine in grey rats. The most active urinary fraction to date was obtained from the crude alcoholic precipitate of anemic urine, by the following procedure: the precipitate was extracted with 0.1 m acetate buffer pH 4.5 and the solution treated with one vol. ethanol. The precipitate which formed showed lower specific activity than the crude extract (Table III-6-7); the supernatant however, on treatment with another 3 vol. of alcohol gave a precipitate whose specific activity is greater than that of the crude extract (Table III-8). Table III-2 shows that the extract of normal rabbit's urine has no effect. The precipitate which forms on treating the crude extract at pH 7 with 1 vol, alcohol has low activity, while that which forms on lowering pH to 4 has as high specific activity as the starting material (Table III-3-4-5).

Discussion. The observations on starved rabbits clearly show that there is a notable decrease in Hb synthesis as reflected by low plasma iron turnover and low incorporation of Fe^{59} in erythrocytes. The analysis of Fe^{59} uptake in red cells at 24 and 48 hr suggests that aside from a decrease in iron consumption by bone marrow, there is also in this case an increase of maturation time of red cell precursors in bone marrow, which makes the estimate of erythropoiesis, as measured by uptake at 24 hr alone, lower than it actually is. The plasma iron turnover in fasted rabbits is as low as the minimum value observed by Bothwell *et al.*(8) in experiments in which X irradiation was used to suppress red cell formation. But in the case of starvation, the depression of erythropoiesis is not a consequence of marrow damage, but probably reflects low Hemopoietine titers(5) and can be reversed by administration of exogenous Hemopoietine, in the form of extract of boiled plasma from phenylhydrazine treated rabbits. The effect of this plasma extract is probably specific, and not the consequence of administration of amino acids, glucose and a few milligrams of protein that are present in the extract, as extract of boiled normal plasma, which conceivably contains similar quantities of these components, has no significant effect. The quantities of Hemopoietine in normal plasma were probably in this case insufficient to obtain effective blood concentration.

The observations on starved rats confirm the finding of depressed erythropoiesis(5) in these animals and show that they are practical assay animals for Hemopoietine. Simultaneous study of Fe^{59} in red cells and liver shows that low erythrocyte Fe^{59} in starved rats is not only a consequence of delayed maturation

of bone marrow precursors, but reflects a change in iron distribution with smaller uptake by the erythroid marrow and a greater proportion of iron going to body stores, as represented by the liver. Hemopoietine injection increases iron uptake by marrow and diminishes that of the stores. The significant linear correlation between Erythrocyte Fe^{59} and $\log_2$ dose, and Erythrocyte/liver Fe^{59} ratio and dose of Hemopoietine, demonstrates the feasibility of carrying out quantitative bio-assays, using the criterion of specific activity in chemical fractionation studies of Hemopoietine. In comparing Fig. 1-a and 1-b, it can be seen that the measurement of E/1 ratio permits detection of smaller amounts of Hemopoietine than does the measurement of Fe^{59} in erythrocytes alone; 2 mg and 4 mg of crude urine alcoholic extract respectively.

If one analyzes that data obtained in normal rats taking into account the dose response relationship shown in Fig. 1, some interesting conclusions can be drawn with respect to assay of Hemopoietine in normal animals. Thus Fe^{59} uptake in normal rats (32%) is seen to correspond to uptake of starved rats injected with 8 mg urinary extract. The increase of Fe^{59} uptake observed by us in normal animals, on receiving 10 mg of an extract of boiled plasma (32.8 to 44.9%) is in the range of what would have been predicted by the dose-response curve of Fig. 1. Thus a dose of 10 mg given to a starved rat increases Fe^{59} uptake 3 fold, while a similar dose given to normal animal increases uptake in only 37%. The gain in sensitivity obtained by using starved animals is manifest.

The presence of "Hemopoietine" activity in urine of bled and phenylhydrazine treated animals, confirms the results of previous experiments(2,3) and accords with the findings of Piliero *et al.*(9), Van Dyke *et al.*(10) and Winkert *et al.*(11) in urine from anemic patients. The fact that extracts of urine from phenylhydrazine treated rabbits are more active than those from bled rabbits is in agreement with the findings that plasma from phenylhydrazine treated animals has more erythropoietic activity than that of bled donors(1,12). The abundance of Hemopoietine in phenylhydrazine treated animals' urine presents a valuable source of material for study, as it is not difficult to maintain rabbits for prolonged periods of time in a state of chronic hemolytic anemia, and obtain several liters of urine from them.

The evidence so far presented by various laboratories points to the protein(13) or polypeptide nature of Hemopoietine, and suggests that it may be classified as a mucoprotein (14,15). The properties here described for Hemopoietine obtained from urine and plasma accord with the properties described for mucoproteins, as chemical analysis of active fractions shows that they contain more than 10 mg total neutral sugar (Orcinol) per 100 mg protein (Biuret).

Summary. 1. Fasted rabbits and rats were used as assay animals for Hemopoietine preparations. Depressed erythropoiesis manifests itself by low plasma iron turnover and uptake of Fe^{59} in erythrocytes in rabbits, and by low erythrocyte and high liver Fe^{59} in rats. 2. Injections of extracts of boiled plasma from phenylhydrazine-treated rabbits increases plasma iron turnover and Fe^{59} uptake by erythrocytes in rabbits, and produces an increase in erythrocyte Fe^{59}, accompanied by a drop of liver Fe^{59} in rats. Uptake of Fe^{59} in erythrocytes is proportional to log dose of Hemopoietine administered, while Fe^{59} erythrocyte/liver ratio is proportional to dose. Active fractions have been prepared from both urine and plasma of anemic rabbits by alcohol fractionation. The active material is non-dialyzable and contains more than 10 mg carbohydrate per 100 mg protein.

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Production of Diabetes Insipidus in the Rat.* (24275)

CARL S. ALEXANDER† (Introduced by Edmund B. Flink)

Veterans Admin. Hospital, and Department of Medicine, University of Minnesota Medical School, Minneapolis

Reports dealing with diabetes insipidus in partially hypophysectomized rats have seldom described operative technics(1-6). This report sets forth in detail the surgical technic of neurohypophysectomy as performed in over 500 animals during the past 5 years, and also more recent experience with hypothalamic lesions produced with a stereotaxic instrument. The water exchange in acute and chronic DI as well as other conditions resulting from such lesions will also be discussed.

Methods. Sprague-Dawley rats of either sex, weighing approximately 200 g, were subjected to neurohypophysectomy by the parapharyngeal approach. Clean but not sterile technic was used. The procedure as described by Smith(7) and Thompson(8) has been modified in 2 important respects: 1) ether is used as the anesthetic because it avoids respiratory depression often seen with barbiturates, and 2) after making a midline neck incision extending from lower jaw to sternal notch, the trachea is incised just below the thyroid cartilage parallel and between 2 rings of tracheal cartilage. A glass tube 4 mm in diameter, 5-6 cm long and tapered to 2 mm at the end serves as a tracheal cannula thru which ether may be administered. Except during actual administration of the anesthetic, it is important to remove ether bottle to avoid CO_2 accumulation and hypoxia. A bloodless plane of dissection between deep strap muscles on the right side of neck can be found by gently retracting with 2 pairs of curved forceps. The digastric muscle is not cut as by Thompson(8) because retraction of the trachea permits adequate exposure of the basisphenoid area of skull. Two curved paper clip retractors are now placed in the plane separating the deep neck muscles dissected on the right side. The exposed basisphenoid area, which can be recognized by the V-shaped muscles inserting along the midline, is freed of its muscle attachment. An important landmark can now be seen—intersection of a longitudinal ridge (crista occipitalis) with the blue transverse cartilage (occipito-sphenoid synchondrosis). Approximately 1 mm rostral to this intersection a hole is drilled and enlarged with a No. 9 dental burr (a smaller size burr may be used to start the hole). Two precautions should be observed during drilling; occasionally a blood sinus in the bone located in rostral portion of hole may be entered, resulting in a brisk oozing of blood which can be stopped only by sustained pressure best applied using #3 round dental pel-

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† Veterans Admin. Clinical Investigator.

TABLE I. Results of Various Procedures Designed to Produce Diabetes Insipidus in the Rat.

Procedure*	No. operated	% mortality	% of survivors developing Acute DI	% of survivors developing Chronic DI
DI	300	20	60	20
C	20	20	30	13
C + DI	200	22	70	25
DI + KS or C + DI + KS	30	50	30	20

* DI, neurohypophysectomy; C, cautery of stalk; KS, hypothalamic lesions.

lets serving as tampons. To some extent this injury in bone sinus can be avoided by centering the hole *at the intersection* in older animals. The second precaution is to avoid drilling completely through the inner table of skull, but to leave a thin shelf of bone which is then carefully removed by a curved, sharp, pointed pick and fine curved forceps. Removal of bone in this manner prevents damage to the gland itself by the burr. The procedure should then be completed with the aid of a binocular dissecting microscope with magnification of 7 to 10X or a loupe. After the bone is completely removed, the underlying tough dural membrane enclosing the pituitary gland is slit down the middle with aid of the pick. The pink anterior lobe immediately bulges into the opening. Complete exposure of the anterior lobe may be afforded by hooking the pick under the free margin of the membrane and pulling each half of the membrane toward the outer margins of the hole as far as possible. Gentle retraction, using 2 picks, one on either side of the anterior lobe, or one pick and a capillary suction pipette, results in a plane of separation permitting division of anterior lobe into 2 halves between which lies the white, fluffy, vermis-like intermediate lobe. The gland of the female is relatively larger and more friable than that of the male. By gentle manipulation of the dissecting needles and curved capillary suction tube, the dark purple-colored posterior lobe can be mobilized, and while restraining the 2 halves of the anterior lobe with the fine curved forceps, suction is abruptly applied to the intermediate and posterior lobes. After tracheal secretions have been aspirated, a single suture consisting of 6-0 silk joined to an atraumatic needle ("eye suture") is placed on either side of the tracheal ring bordering the tracheal opening. Trial closure of the tracheostomy is carried out before the tie is secured. Before suturing, skin margins are freed from underlying connective tissue to allow more space for accumulation of any serous fluid. Ballooning of wound after closure indicates an air leak either from trachea or from fistula in the hypopharynx. The former can easily be repaired but the fistula is difficult or impossible to repair; such animals are best discarded since death will most certainly occur early in the postoperative period. Final step in the procedure is aspiration of blood and mucus from nares and oropharynx. With skill entire procedure takes 15-20 minutes. Requirements for postoperative care are simple: Room temperature should be 78-80° F, animal in individual cage for at least 10 days, and regular stock diet. *Use of cautery.* The technic is the same as described above with certain modifications. After separation of the anterior lobe from the neurohypophysis, the exposed end of a No. 26 varnished copper wire electrode is applied to the proximal end of stalk. The indifferent electrode is placed in the rectum. A current of 3 to 5 milliamperes‡ for 15 to 18 seconds is applied to the stalk without damaging the neighboring anterior lobe. Usually this results in appearance of small bubbles in the fluid bathing the electrode and sometimes even an eschar. The neurohypophysis is then gently dissected free from the coagulum and aspirated as described above. *Use of stereotaxic instrument.*§ The technic employed has been similar to that described by McCann(9) with minor modifications. The aim is to destroy the supraoptic and paraventricular nuclei producing ADH.

Results. A summary of results covering operative mortality and the development of acute and chronic DI in 550 rats is given in

‡ May be furnished by a D.C. Heathkit regulated power supply with a milliammeter and voltmeter in the circuit.

§ The instrument and atlas of cross-sectional anatomy of the rat brain are available from Johnson Instrument Co., Berwyn, Ill.

Table I. In this study DI is considered present when urine output is 3 or more times that of normal rats of comparable weight on a Fox-chow, water *ad lib.* diet. Approximately 6% of animals operated excreted 100 ml or more of urine/day at some time during postoperative period (Table II). In ½ the group with transient DI a residual defect in urine concentration persisted despite urine output within the range of normal to 3 times normal. The remainder, ⅙ of the group, returned to a normal condition. Chronic DI developed more frequently after hypothalamic lesions than with other technics, and the highest urine outputs occurred in these animals. But this advantage was more than offset by higher mortality and the work involved in performing an extra procedure (hypothalamic lesions in normal rats are less satisfactory). Three such animals had sustained polyuria, excreting 150-240 ml of urine for several weeks and in 2 of the 3 obesity due to hyperphagia was also present.

Early postoperative course. Urine output may be quite variable for the first few days. Passage of dilute urine within hours after surgery occurred even before animals began to drink water. Despite the extreme degree of fluid exchange seen early in some of these animals, there was a sudden or gradual decline in rate of water exchange to a level only 2 to 3 times normal. However, in about 50% of such animals 2 permanent abnormalities remained: 1) urine specific gravity persisted at lower than normal value and did not reach that of controls even after large isolated doses of pitressin, and 2) when the animal was offered 1% NaCl in place of water as sole drinking fluid there was a sharp increase in level of fluid exchange far exceeding the response of a normal rat to this maneuver (Fig. 1). An atypical course in one animal charactreized by unexplained extreme fluctuations in fluid exchange is illustrated in Fig. 2.

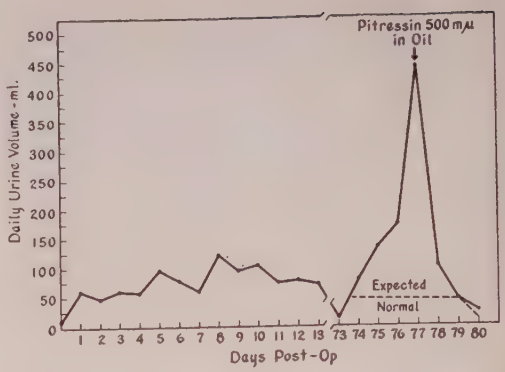
Chronic diabetes insipidus. One-third of

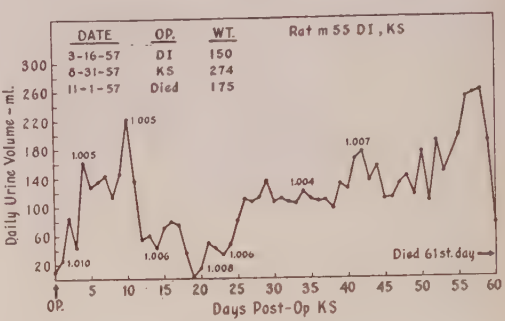
TABLE II. Excretion of 100 ml or More of Urine per Day (1 Day or Longer) in Survivors of Various Operative Technics.

Procedure*	No.	Urine output (ml/day) Avg	Range	Sp. gravity Avg	Range
DI	18	148	105-170	1.007	1.005-1.008
C	1	182		1.009	
C + DI	11	150	113-175	1.007	1.003-1.012
C+DI+KS	2	222	186-258	1.004	1.003-1.005
	2†	178	161-166	1.004	1.012-1.025

* See footnote, Table I.

† Polyuria was associated with hyperphagia and obesity.





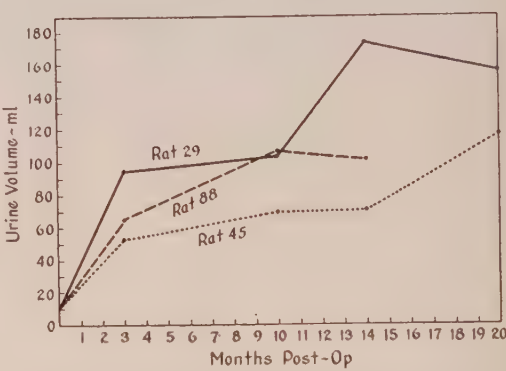


FIG. 1 (top). Pattern of fluid exchange in neurohypophysectomized rat. Exaggerated response to drinking 1% NaCl substituted for water on 73rd postoperative day.

FIG. 2 (center). Unusual course in fluid exchange in rat with neurohypophysectomy and electrolytic lesions of the hypothalamus.

FIG. 3 (bottom). Avg daily urine output on stock diet in 3 neurohypophysectomized rats.

all animals with acute DI developed chronic DI. That diabetes insipidus can be both severe and permanent is illustrated in Fig. 3 which shows average daily urine output over a long period of 3 animals subjected to neurohypophysectomy. Diabetes insipidus animals with severe polyuria generally do not show signs of anterior pituitary deficiency such as inactivity, weakness, fur changes or testicular atrophy.

Other postoperative syndrome. Electrolytic lesions made with the stereotaxic instruments produced a variety of other sequelae deserving mention. In contrast to the syndrome characterized by viciousness, hyperphagia, polydipsia, polyuria and eventual obesity, other animals with brain lesions made in the same manner refused to eat, drink or move about the cage and died within a few days. Life could not be prolonged beyond an extra few days by administration of DCA, cortisone or parenteral glucose and saline. In some rats arching of spine and ataxia on forced movement was noted. Others were extremely agitated as soon as they recovered from anesthesia and circled the cage incessantly as if trying to escape. They, too, ate and drank little or nothing and within a few days died in a state of apparent exhaustion. It is important to note that many of these animals excreted dilute urine early postoperatively even in complete absence of food or water intake. Pitressin administration produced an increase in urine concentration without appreciably affecting the volume.

Discussion. The existence of permanent DI in the rat has been questioned by some workers(10,12), but others have observed DI of long duration(1,4,5). Diabetes insipidus itself has been arbitrarily defined and therefore the reported duration and severity may differ from one report to another. Compared to values reported by others(1,4,5,6), it is evident that excretion of urine exceeding 100 ml/day by animals weighing 250-300 g reflects severe ADH deficiency. Assuming glomerular filtration rate (GFR) of 0.6 ml/min/100 g body weight for the rat(13), excretion of 100 ml of urine/day by a 250 g rat amounts to 4.6% of total plasma filtered. Assuming for man, 187 liters of plasma is filtered/day, the same fraction excreted would produce 8.6 liters/day which approximates the output of the human with DI of average severity. The highest daily urine output ever recorded in these DI rats (258 ml, corresponding to 11.9% of the total plasma filtered) would be comparable to a urine output in the human of over 21 liters, a figure rarely reported even in most extreme cases of human DI. Inasmuch as maximum concentrating ability of rat kidney is greater than the human kidney (2000 mosm/l *vs.* 1500 mosm/l(14), respectively), urine output in these rats with severe DI is even more impressive.

It is clear from these postoperative results and from the work of others that amount of urine excreted depends not only on ADH, but also on rate of water and solute excretion, which in turn depends on the integrity of the appetite(15-17) and drinking centers(18). Damage to these centers even in absence of ADH leads to oliguria and hyposthenuria and may explain the unexpected appetite for salt (Fig. 1) and fluctuating thirst (Fig. 2). It is well known that diuresis induced in DI subjects by isotonic saline can be interrupted by pitressin. Degree of response is a function of pitressin dosage and rate of solute excretion (21), and Jacobson and Kellogg(22) have even shown that pitressin can inhibit diuresis induced in normal rats with hypertonic saline (0.23 M NaCl). The prompt antidiuresis caused by a relatively large dose of pitressin (Fig. 1) may also be explained in this way.

Summary. Experience with several technics for producing experimental diabetes insipidus (DI) in 550 rats has been described. These include neurohypophysectomy, cautery of stalk, and electrolytic lesions of the hypothalamus produced with aid of a stereotaxic instrument.

I am grateful to Dr. E. B. Flink for helpful suggestions and review of the manuscript.

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Lipemia in the Rabbit Following Injection of Pituitary Extract.* (24276)

DANIEL RUDMAN† AND FLOYD SEIDMAN (Introduced by David Seegal)
Columbia Univ. Research Divvision, Goldwater Memorial Hospital and Dept. of Medicine, Columbia University, N. Y. City

It has been recognized that the pituitary gland plays a role in regulation of lipid metabolism. Injection of pituitary substances into experimental animals has been reported to produce the following changes: (A) Decrease in fat content of carcass and adipose tissue, and increase in fat content of liver(1, 2); (B) Increase of ketone bodies in blood and urine(2,3,4); (C) Depression of respiratory quotient(5); (D) Increase in plasma content of unesterified fatty acids(6). These effects appear to result from mobilization of lipid from fat depots, with subsequent deposition of fat in the liver, and increased oxidation of fatty acids associated with accumulation of ketone bodies(2,7,8). Increase in liver fat content has been observed following injection of anterior pituitary extract, and of various anterior pituitary fractions, including adrenotropic, growth, and thyrotropic preparations (9,10). Increase in plasma unesterified fatty acids has been observed after injection of growth hormone(6). A further effect of pituitary material upon circulating lipids has been described. Evans(11), Munoz(12), Baumann(13), and Campbell(14) observed lipemia in dogs and rabbits made diabetic by repeated injections of anterior pituitary extract or of purified growth hormone. Greenbaum(15) described a transitory increase in plasma triglyceride of rats receiving daily injections of growth hormone. Seifter(16) reported that a dialyzable material obtained from posterior lobe of hog pituitary is inhibitor of post-heparin plasma clearing factor and causes an increase in plasma lipids of animals with liver damage, or of animals fasted for 20 hours. The present study was undertaken to determine the effect of injections of crude pituitary extract and of various purified pituitary fractions, upon serum lipid pattern of the rabbit.

Methods. Male rabbits weighing 2.5 to 4 kg were used. They were fed Purina rabbit chow with the exception that in experiments where blood sugar determinations were made

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† Senior Research Fellow, N. Y. Heart Assn.

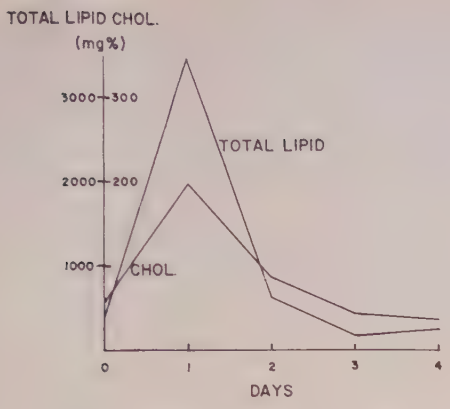

FIG. 1. Time curve of lipemic response following single subcut. inj. of extract of 100 mg lyophilized whole hog pituitary gland.

the food was withheld 3 hours before bleeding. Analytical methods are described in the following references: serum cholesterol(17), serum total lipid(18), blood sugar(19), plasma unesterified fatty acids(20), paper electrophoresis of serum(21). Samples of lyophilized or acetone-desiccated whole pituitary gland were pulverized in mortar, then extracted for 5 hours at 5°C with M/10 Na_2HPO_4 (125 ml for 1 g of pituitary powder). The suspension was clarified by centrifugation and filtration. Approximately 40% of the pituitary material is extracted by alkaline solution.‡

Results. A single subcutaneous injection of the extract from 100 mg of lyophilized whole hog pituitary caused gross lipemia in 16 of 20 rabbits. The increase in serum cholesterol and total lipid values was apparent 6 hours after injection. It reached a maximum in 18 hours and usually disappeared within 48 hours (Fig. 1). Electrophoretic study of serum in 5 rabbits following a single subcutaneous injection of lyophilized hog pituitary extract showed that most of the newly appearing serum lipid had little or no mobility and thus consisted largely of chylomicra (Fig. 2). In 5 other rabbits which received a daily injection of pituitary extract for 3 days, the lipemia was maintained during entire period and on the 4th day electrophore-

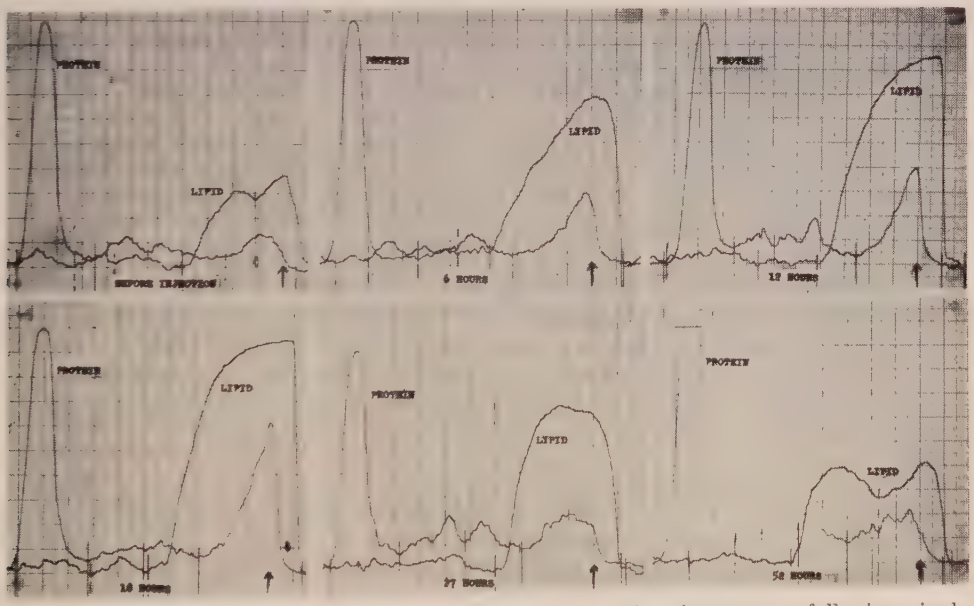

FIG. 2. Electrophoretic patterns of sera obtained during lipemic response following single subcut. inj. of extract of 100 mg of lyophilized whole hog pituitary gland. Serum was obtained before inj., and 6, 12, 18, 27 and 52 hr after inj. Arrows indicate origin in each tracing.

‡ Recent experiments show that more efficient extraction is obtained with 0.1N NaOH for 15 minutes at 5°C.

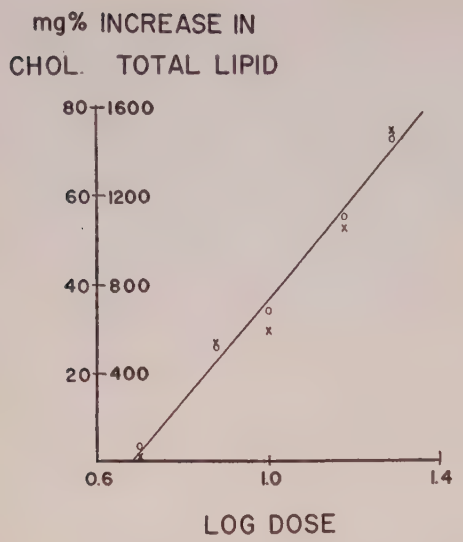

FIG. 3. Relationship of response to dose. Vertical axis: increase in total lipid and cholesterol values of serum 18 hr after single subcut. inj. of extract of lyophilized whole hog pituitary gland. Horizontal axis: logarithm of dose (mg gland/kg body wt). Each point represents avg increase in serum total lipid or serum cholesterol of 6 rabbits. Crosses represent increase in total lipid. Open circles represent increase in cholesterol.

sis showed a prominent beta-lipoprotein peak in addition to the chylomicron peak. A change in alpha lipoprotein was not observed. An additional finding in 3 of 5 rabbits during the first 24 hours after injection, was an increase in a protein component which had no electrophoretic mobility (Fig. 2). This may represent gamma-globulin, or a protein associated with the chylomicra. A 2 to 3 fold increase in serum unesterified fatty acids was found in 4 rabbits whose unesterified fatty acids were determined before, and 18 hours after, a single injection of hog pituitary extract (Table I). Blood sugar levels were determined in 20 rabbits during control and lipemic periods and showed no significant change. In 5 of these rabbits, in which intravenous glucose tolerance tests (0.5 g glucose/kg body weight) were done during control and lipemic periods, there was no detectable change in glucose tolerance curve during the lipemic period.

Dose-response relationship was studied in 5 groups of 6 rabbits which received doses of lyophilized whole hog pituitary extract representing 5, 7.5, 10, 15 and 20 mg of gland/kg body weight respectively. The response, R, (increase in serum cholesterol or serum total lipid 18 hours after a single injection), showed a linear relationship to the logarithm of dose, D, (mg of pituitary gland/weight of rabbit in kg). This relationship is shown in Fig. 3. The dose-response equations were: $R_{cholesterol} = 120\ LOG_{10}\ D - 85$; $R_{total\ lipid} = 18\ (120\ LOG_{10}\ D - 85)$.

Dose-response curves were also obtained with several other pituitary samples. Lipemia-producing activity of lyophilized hog whole pituitary gland, and of lyophilized hog anterior pituitary gland, was 3 to 4 times greater than that of acetone-desiccated preparations of whole hog, sheep or beef pituitary. Frozen human pituitary glands obtained at autopsy, through the courtesy of Dr. Sigmund L. Wilens, had an activity comparable to that of lyophilized whole hog pituitary glands. Dose-response curves obtained with samples of lyophilized anterior and posterior hog pituitary lobes indicate that the activity of a single anterior lobe is about 7 times greater than that of a single posterior lobe.

Crude anterior pituitary extract is known to contain 6 fractions with distinct hormonal activities. The lipemia-producing activity of

TABLE I. Effect of Hog Pituitary Gland Extract upon Serum Unesterified Fatty Acids. Each rabbit received a single injection of extract representing 60 mg of lyophilized whole hog pituitary.

Rabbit	Unesterified fatty acids (μeq/l)		Serum cholesterol (mg %)		Serum total lipid (mg %)	
	Before inj.	18 hr after inj.	Before inj.	18 hr after inj.	Before inj.	18 hr after inj.
CAL.	190	630	56	162	400	2080
RA.	220	600	55	116	380	2220
K.	180	670	62	152	410	2180
ILL.	150	390	66	128	440	1180

TABLE II. Effect of Crude Pituitary Extract and of Various Anterior Pituitary Fractions upon Serum Lipids of Rabbit. Serum total lipid was measured before, and 18 hours after, a single subcutaneous injection of each preparation.

Preparation	No. of rabbits	Dosage	Change in serum total lipid (mg %)
Oxycel ACTH (Armour R216-170A)	3	120 I.U. (4.8 mg)	−10‡ (+20, +220, −265)§
$\alpha_1 + \alpha_2$ corticotropin (Lederle S1079-31)	5	270 " (3 ")	0 (+110, 0, +10, −50, −80)
$\gamma_1 + \gamma_2$ corticotropin (Lederle S1079-45)	4	270 " (3 ")	+10 (+40, +130, −120, −20)
Growth hormone (Armour R50109)	5	12.5 mg	+10 (−120, +160, −30, +110, −70)
Idem	4	25 "	+250 (+110, +60, +30, +790)
Lactogenic hormone (Armour R10109)	4	12.5 "	+30 (+50, +160, +10, −120)
Thyrotropic hormone (Armour S3112)	3	10 I.U.	+10 (+100, −80, +15)
Follicle-stimulating hormone (Armour 19911)	4	25 mg	+30 (+20, +110, −60, +30)
Luteinizing hormone (Armour S10407)	4	12.5 "	+40 (+80, −50, +115, +10)
Lyophilized whole hog pituitary extr.	6	9 " *	+540 (+570, +380, +1180, +1150, −30, −40)
Idem	5	24 " †	+1450 (+1680, +1810, +740, +1790, +1250)

* Represents soluble pituitary material extracted from 23 mg of lyophilized pituitary gland.
† *Idem* 60 mg
‡ Avg serum total lipid change of group.
§ Total lipid change of each rabbit in group.

these purified anterior pituitary fractions was compared with activity of crude extract (Table II). The extract derived from 60 mg of lyophilized whole hog pituitary gland contains 24 mg of soluble pituitary material; a single injection of this amount of crude extract consistently produces lipemia in the rabbit (Table II). Each of the 6 purified anterior pituitary fractions was tested at a dosage level which is 5 or more times greater than the amount of hormone which is contained in 60 mg of whole hog pituitary gland. Follicle-stimulating, luteinizing, lactogenic, and thyrotropic hormones had no effect upon rabbits' serum lipids at corresponding dosage of 10 mg, 12.5 mg, 12.5 mg, and 10 International Units. Growth hormone had no effect at dose of 12.5 mg, but at dose of 25 mg caused a significant increase in serum total lipid content in 1 of 4 rabbits. Oxycel-purified ACTH had no effect in a dosage of 120 I.U. "Peptide" ACTH preparations, furnished by Dr. Paul H. Bell of Lederle Laboratories, had no detectable effect upon the rabbits' serum lipids in dosages containing 270 I.U. of ACTH activity.

The lipemia-producing material in the extract of lyophilized whole hog pituitary gland showed these properties: (1) Activity was unchanged when the extract was stored 1 week at −15°C. (2) Activity was destroyed by heating at 100°C for 30 minutes, at pH 4.5 or 7.0. (3) Extracts which had been dialyzed 48 hours against running tap water showed no detectable loss of activity. No activity was present in the dialysate obtained by equilibrating a suspension of 300 mg of powdered gland through viscose tubing against 30 ml M/10 Na_2HPO_4 at 4°C for 24 hours. (4) All activity of the crude extract could be recovered from the precipitate obtained by addition of 2 volumes of saturated ammonium sulphate at pH 6.5. (5) When spun in ultracentrifuge at 40,000 rpm for 20 hours, the active material did not sediment or float at specific gravity 1.22. All activity was concentrated in the lower fraction after ultracentrifugation at 40,000 rpm for 20 hours at specific gravity 1.005. (6) The active ma-

terial was completely adsorbed by calcium phosphate gel at pH 4.0. It was eluted from the gel by M/10 sodium phosphate buffer at pH 10. These properties are consistent with the hypothesis that the active material is a protein.

Discussion. The results indicate that crude extracts of lyophilized whole or anterior pituitary glands, derived from men, hogs, sheep, or cattle, contain a substance which causes lipemia in the rabbit. The relationship of the lipemia effect to increase in liver fat content and to increase in production of ketone bodies, described by other investigators, following injections of pituitary preparations, remains to be studied. All 3 effects may result from release of stored fat from fat depots.

Six different hormones are present in the anterior pituitary gland: *viz.* adrenotropic, growth, lactogenic, thyrotropic, follicle-stimulating, and luteinizing hormones. Purified preparations of these hormones had no significant effect upon serum lipids of the rabbit at dosages which contained at least 5 times the amount of hormone present in a lipemia-producing dose of the crude extract. These findings suggest either that the crude extract of the gland contains a lipemia-producing substance which is distinct from the recognized pituitary hormones, or that 2 or more pituitary hormones act in a synergistic manner upon the rabbit's serum lipids.

Summary. An extract prepared from lyophilized hog anterior pituitary gland produces lipemia in rabbits. A similar effect is produced by whole pituitary glands from men, hogs, sheep, and cattle. Activity of the crude extract of lyophilized glands is considerably greater than can be accounted for by the presence of any of the purified anterior pituitary fractions so far tested. This suggests that the lipemia-producing substance is distinct from the recognized pituitary hormones, or that 2 or more pituitary hormones act in a synergistic manner upon the rabbit's serum lipids.

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Inhibitory Effect of a Serum Protein on Development of Opalescence in Lipoproteins Altered by Lecithinase C.* (24277)

ELMA KRUMWIEDE (Introduced by Ann G. Kuttner)
Department of Pediatrics, N. Y. University-Bellevue Medical Center, N. Y. City

The production of opalescence by *Cl. welchii* in broth containing human sera was reported independently by Nagler(1) and by Seiffert(2). MacFarlane and Knight(3) demonstrated that the reaction was due to the presence of lecithinase C in the α toxin of *Cl. welchii* and that development of acid soluble phosphorus in human sera and egg yolk could be used as a measure of enzyme activity. In contrast to published data on the action of *Cl. welchii* toxin on human sera, reports of several observers on horse sera yielded contradictory results(4). The effect of lecithinase C on horse sera, therefore, was reinvestigated.

Methods and materials. Methods for lipid extraction, cholesterol determinations and electrophoretic studies have been described (5). Lipid phosphorus and acid-soluble phosphorus in trichloroacetic acid filtrates (9 parts trichloroacetic acid, 1 part test material) were determined by the method of Stewart and Hendry(6) as modified by Ahrens (personal communication). The specific gravity of material for ultracentrifugation was raised with a saturated solution of KBr in 8.5% NaCl. After 20 hours centrifugation at 40,000 rpm in a Spinco preparative ultracentrifuge, the infranates and supernates were removed with capillary pipettes and dialyzed against 8.5% NaCl to remove KBr. Crude α_1 lipoprotein fractions were prepared by a modification of the method of Macheboeuf(7). One volume of horse serum† and one volume of 8.5% NaCl were stirred with 2 volumes of a saturated solution of ammonium sulfate and filtered. The filtrate was adjusted to pH 4.5 with 1N HCl at 30°C. The precipitate which formed was collected by centrifugation. After washing with 8.5% NaCl to remove all ammonium sulfate, it was emulsified in 8.5% NaCl (50 ml/L of serum). 1N NaOH was added until material dissolved and the pH adjusted to 7.0 with 1N HCl. This serum fraction contained α_1 lipoproteins and a protein which was shown by ultracentrifugation and lipid analysis to be lipid-free, hereafter referred to as L-FP. Borate buffer which was used to dilute all material was prepared by adding sufficient M/20 borax to M/5 boric acid in M/20 NaCl to bring the pH to 7.0. Filtrates of *C. perfringens* (*Cl. welchii*)‡ used as a source of lecithinase C contained phosphate buffer which was removed by precipitation with $CaCl_2$. To 5 ml of filtrate, 1 ml of 0.5 M $CaCl_2$ and 4 ml of borate buffer, 1N NaOH was added until no further precipitate formed. After overnight refrigeration it was centrifuged and the precipitate discarded. The supernate contained traces of phosphorus and 77 mg% Ca. Lecithinase was not standardized(3). Preliminary studies showed that maximum amount of acid soluble phosphorus released from lipoprotein fractions with equal lipid phosphorus content was dependent on unknown factors as well as concentration of lecithinase. Therefore, the amount of lecithinase necessary for production of maximum hydrolysis was determined for each substrate by preliminary titration. In tests where development of opalescence only is reported description of these titrations is omitted. Merthiolate in a final concentration of 1-10,000 was added to all materials.

Results. Effect of lecithinase C on horse serum. One ml of horse serum and one ml of a 1-16 dilution of lecithinase C in borate buffer (pH 7.0) were incubated overnight at 37°C. The results are presented in Table I. There was no correlation between development of acid soluble phosphorus and production of opalescence in sera in which the phos-

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† Normal horse serum was generously supplied by Bureau of Laboratories, Dept. of Health, N. Y. City.

‡ *C. perfringens* filtrates were kindly supplied by Lederle Laboratories.

TABLE I. Effect of Lecithinase C on Horse Sera.

Phospholipid hydrolyzed ASP* (%)	Appearance
0	clear
0	"
0	"
70	"
89	"
82	slightly opalescent
80	milky

* ASP = Acid soluble phosphorus.

Percentages were obtained by dividing ASP content by original lipid P content.

pholipids were hydrolyzed by lecithinase C. These results indicate that a number of variable factors influence the effect of lecithinase C on phospholipids of horse sera. Since horse sera contain relatively large amounts of a_1 lipoproteins, the effect of lecithinase C on crude horse serum a_1 lipoprotein fractions was studied.

Effect of lecithinase C on crude a_1 lipoprotein fractions. Ten crude horse serum a_1 lipoprotein fractions were incubated overnight at 37°C with varying concentrations of lecithinase C. Concentration of lipid phosphorus in the fractions varied from 0.040 to 0.080 mg/ml. Percentage of phospholipid subject to hydrolysis by lecithinase C in different fractions varied from 65-90%. Four of the 10 fractions remained clear and 6 developed traces of opalescence. There was no correlation between development of opalescence in lipoprotein fractions and sera from which they were prepared.

Electrophoretic patterns. Paper electrophoretic patterns(5) showing the effect of hydrolysis of phospholipids in a crude a_1 lipoprotein fraction by lecithinase C are shown in Fig. 1. The most marked changes in the electrophoretic patterns resulting from hydrolysis of phospholipids by lecithinase C are a decrease in amount of L-FP and a slight increase in electrophoretic mobility of the a_1 lipoproteins.

Trypsin digestion of crude a_1 lipoprotein fractions. Since free lipids do not generally move in an electrophoretic field, the electrophoretic patterns of the crude a_1 lipoprotein fraction treated with lecithinase C (Fig. 1) suggest that cholesterol and the diglyceride portion of hydrolyzed phospholipids remain bound to protein. To determine whether these lipids were protein-bound, the results of ether extraction of lipoprotein fractions before and after enzyme action were compared. Only traces of lipid could be extracted with ether before and after treatment with lecithinase C. To obtain further evidence that lipids were still bound to protein, the effect of ether extraction following hydrolysis of the proteins with trypsin was investigated. Three crude a_1 lipoprotein fractions were incubated overnight at 37°C with lecithinase C (1.5 ml), with 1-400 trypsin§ (2.0 ml) and both lecithinase and trypsin. Volumes were adjusted to 5 ml with borate buffer. The lipid content of the fractions, amount of phospholipid hydrolyzed and ether extractable lipids are shown in Table II. Hydrolysis of the proteins by trypsin liberated a high percentage of lipids, while hydrolysis of the phospholipids by lecithinase C liberated little or no lipid. Although no chemical analyses were done to identify the lipids in the fat globules seen after hydrolysis of both the phospholipids and protein, they appear to represent the diglyceride portion of the hydrolyzed phospholipids. Bragdon's method(8) for determination of neutral fat based on reduction of potassium dichromate by lipid carbon with subsequent correction for phospholipid and cholesterol content was used to determine diglyceride content. Low lipid phosphorus values precluded calculations of amount of dichromate reduced by phospholipids. Amount of dichromate reduced therefore is given as a crude measure of lipid content. These experiments showed not only that lipid-protein linkages were present after hydrolysis of the phospholipids by lecithinase C but also that more phospholipid could be hydrolyzed when the proteins were hydrolyzed by trypsin.

Ultracentrifugation. The a_1 lipoproteins and L-FP in 4 crude lipoprotein fractions were separated by ultracentrifugation at specific gravity 1.175. The supernate contained a_1 lipoproteins. The infranates of 3 contained

§ Salt free trypsin, Worthington Biochemical Co., Freehold, N. J. 25 mg in 10 ml distilled H_2O.

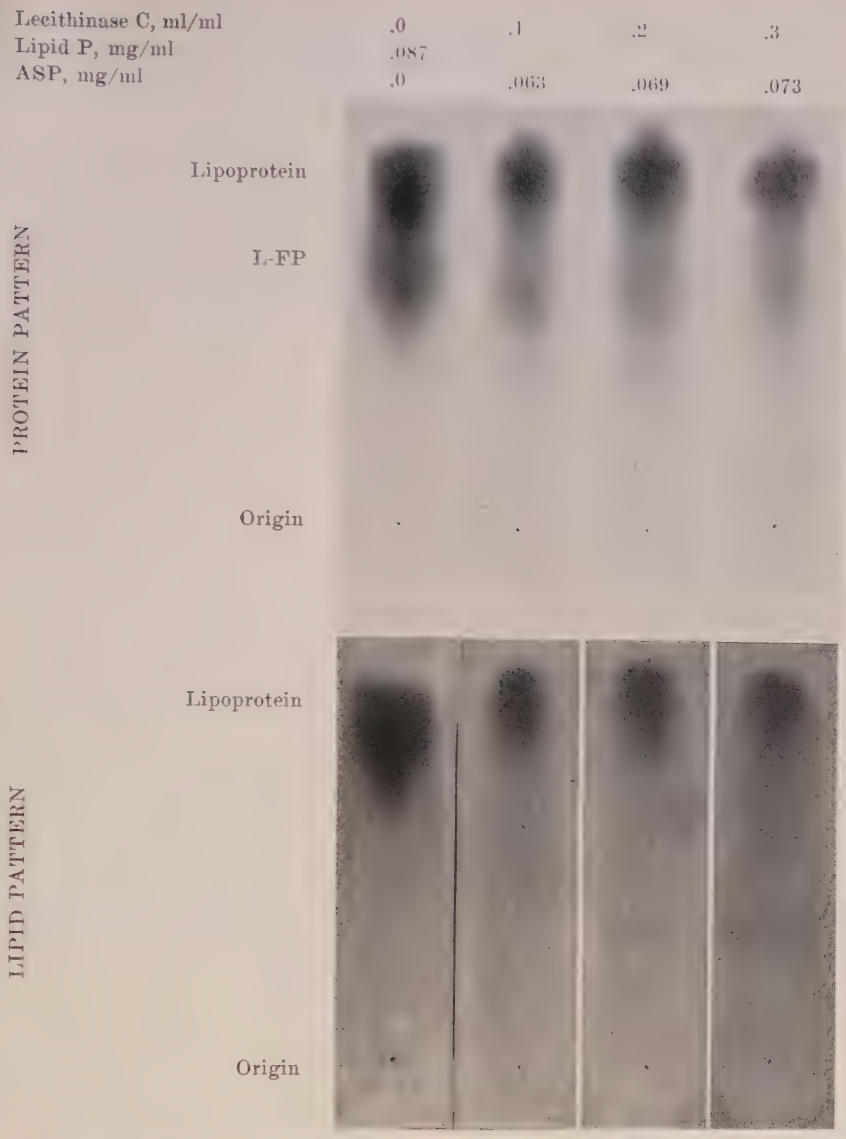

FIG. 1. Decrease in lipid-free protein accompanying hydrolysis of phospholipids in a crude a_1 lipoprotein fraction by lecithinase C. Final volume of each test was brought to 1.0 ml with borate buffer and incubated overnight at 37°C.

only L-FP. The infranate of the fourth contained L-FP and traces of albumin which had not been detected in electrophoretic patterns of the original material. The 4 a_1 lipoprotein fractions were incubated overnight with lecithinase C. Three became opalescent and a precipitate formed in the fourth. To determine whether lipids were still bound to protein 0.8 ml samples were incubated overnight with 0.2 ml of 1-200 trypsin. All developed a thick creamy appearance suggesting that lipids were freed when the protein was hydrolyzed.

Effect of L-FP on development of opalescence in lipoprotein fractions. An a_1 lipoprotein fraction was treated with lecithinase C after addition of decreasing amounts of the L-FP fraction containing albumin, L-FP markedly reduced precipitation and development of opalescence but did not influence

TABLE II. Lipids in Ether Soluble Form and Appearance of Crude α_1 Lipoprotein Fractions Treated with Lecithinase C, Trypsin, and Both Lecithinase and Trypsin.

Lot		Lipids in ether soluble form — After treatment with —				Increase in ASP	
	Total lipid content before treatment	Control	Lecithinase	Trypsin	Lecithinase and trypsin	Lecithinase	Lecithinase and trypsin
	mg/ml						
1. Lipid P	.092	trace	trace	.055	trace	.084	.089
Cholesterol	1.670	.16	.11	1.07	1.61		
Dichromate reduced	85.7	5.0	4.6	33.3	41.9		
Appearance before ether extr.		clear	clear	opalescent	visible fat		
2. Lipid P	.088	trace	trace	.034	trace	.078	.081
Cholesterol	1.710	.16	.16	1.4	1.4		
Dichromate reduced	67.7	trace	trace	41.7	52.0		
Appearance before ether extr.		clear	clear	opalescent	visible fat		
3. Lipid P	.085	trace	trace	.077	trace	.071	.080
Cholesterol	1.369	.186	.221	.945	.966		
Dichromate reduced		5.0	11.7	44.3	46.7		
Appearance before ether extr.		clear	clear	opalescent	visible fat		

liberation of acid soluble phosphorus. To rule out the possibility that L-FP might be exerting a non-specific effect, a freshly prepared α_1 lipoprotein fraction (sp. gr. 1.175) was treated with lecithinase C in the presence of 5% bovine albumin,‖ concentrated human serum albumin,¶ 5% casein, egg white and the L-FP fraction containing albumin. Each test contained 0.2 ml of lipoprotein (439 mg/ml lipid phosphorus), 0.1 ml of lecithinase C. 0.2 ml of test material and borate buffer to 1 ml. A control containing lipoprotein and lecithinase was included as were controls to determine effect of lecithinase on test materials. Additional controls of test materials and lipoprotein were set up. The results showed that the effect of L-FP on development of opalescence is specific. Only a trace of opalescence was observed in the presence of L-FP while marked opalescence and a precipitate were seen in tests containing only lipoprotein and lecithinase. Marked opalescence but no precipitate developed in the presence of egg white suggesting that egg white may contain L-FP.

Several pools of human plasma were fractionated. The yield of lipoprotein, which was smaller than that from horse serum, was greatly reduced when the material was washed sufficiently to eliminate albumin by removal of all ammonium sulfate. One human fraction containing visible fat globules and albumin as well as lipoprotein and L-FP was used in the following experiments. Fat globules were first removed by ultracentrifugation at specific gravity 1.075 and the α_1 lipoproteins then separated from albumin and L-FP at specific gravity 1.175. Opalescence and a precipitate were observed when human lipoprotein was incubated with lecithinase C. No precipitate formed and opalescence was markedly reduced by addition of either horse or human L-FP.

Standardization of L-FP on basis of nitrogen content. Human serum to which 4 concentrations of 3 albumin free lots of horse serum L-FP had been added were treated with lecithinase C. The results are shown in Table III. Development of opalescence was greatly reduced in presence of L-FP. Correlation between nitrogen content and suppression of opalescence was not absolute suggesting that the L-FP samples were not pure.

Influence of L-FP on development of opalescence in human sera. Nine human sera

‖ Bovine albumin powder, Armour Laboratories.

¶ Normal serum albumin (human) kindly supplied by American Red Cross.

TABLE III. Effect of L-FP on Development of Opalescence in Human Serum.

Lot L-FP	mg/ml L-FP nitrogen .26	.21	.16	.10	Lecithinase control	Serum control
I	±	±	±	2+	4+	—
II	±	±	±	+	4+	—
III	+	+	+	2+	4+	—

Each test contained 0.25 ml of serum (0.084 mg/ml lipid phosphorus), 0.05 ml of 1-5 lecithinase, and L-FP; lecithinase control, serum and lecithinase. All volumes were adjusted to 0.6 ml with borate buffer. Plus signs indicate degree of opalescence.

were treated with lecithinase C with and without addition of L-FP. Tests contained 0.5 ml of serum, 0.1 ml of lecithinase and 0.3 ml of L-FP (Lot I, final nitrogen content 0.16 mg/ml). L-FP was omitted from the lecithinase controls. All volumes were adjusted to 1.2 ml with borate buffer and incubated overnight at 37°C. The results are shown in Table IV. Addition of L-FP markedly reduced development of opalescence and milkiness resulting from hydrolysis of phospholipids by lecithinase C in 8 of 9 sera. Reduction of opalescence in serum (M.B.) which had high phospholipid and cholesterol values was only slight. To determine whether addition of larger amounts of L-FP would further reduce the milkiness in this serum, it was treated with lecithinase C after the addition of L-FP (Lot II, Table III). With a final nitrogen concentration 0.019 mg/ml this lot markedly reduced development of opalescence. Two sera (P.N. and P.B.) were incubated with lecithinase C after addition of human L-FP. No opalescence developed although as much phospholipid was hydrolyzed as in lecithinase treated controls.

Discussion. The development of opalescence or milkiness in sera treated with lecithinase C has generally been regarded as evidence of hydrolysis of phospholipids with subsequent liberation of lipids from lipoproteins (3,9,10). The work reported here was undertaken because of conflicting reports on development of opalescence in horse sera(4). It was found that hydrolysis of phospholipids in horse sera and crude horse serum lipoprotein fractions does not always lead to development of opalescence. Electrophoretic patterns of lecithinase treated crude lipoprotein fractions showed that L-FP (a lipid-free protein) present in the fractions is reduced when phospholipid is hydrolyzed. It was then shown that L-FP from both human and horse sera suppressed opalescence in whole human sera as well as in α_1 lipoproteins obtained by ultracentrifugation of crude horse and human fractions. Electrophoretic patterns also suggested that hydrolysis of the phospholipids altered but did not destroy the lipoproteins, *i.e.*, free the lipids from the protein. Further evidence of lipid-protein linkage after hydrolysis of the phospholipids in lipoproteins was obtained by ether extraction and trypsin digestion. The cholesterol and the diglyceride portion of the phospholipids remained bound to protein suggesting that phospholipids in α_1 lipoproteins are bound to protein through fatty acids by secondary valences(11,12). These findings appear to be at variance with those obtained by others. However, after high speed centrifugation to separate the "freed lipids" Crook (9) found that the fatty layer was ⅓ protein

TABLE IV. Effect of Horse Serum L-FP on Development of Opalescence in Human Sera Treated with Lecithinase C.

Serum	Lipid P, mg/ml in test	Effect of lecithinase C: Increase in ASP, mg/ml	Effect of lecithinase C: Opalescence	Effect of lecithinase C & L-FP: Increase in ASP, mg/ml	Effect of lecithinase C & L-FP: Opalescence
P.S.	.037	.028	4+	.029	± ?
P.B.	.033	.028	4+	.027	± ?
P.N.	.038	.031	3+	.029	±
M.S.	.034	.028	4+	.029	±
R.P.	.035	.031	4+	.030	+
L.L.	.034	.029	milky	.029	+
J.W.	.038	.031	very milky	.027	+
J.H.	.042	.036	*Idem*	.033	+
M.B.	.049	.043	"	.043	milky

and Peterman(10) found a 5% nitrogen loss in the infranate. These results as well as those reported here suggest that lipid-protein bonds in lipoproteins are not necessarily destroyed by the action of lecithinase C. They suggest rather that solubility of the lipoprotein is altered when phosphorylcholine is freed from the phospholipid.

Although MacFarlane and Knight(3) suggested that the integrity of the lipoprotein complex might be dependent on lecithin, they pointed out that development of turbidity is a secondary reaction. At pH 5.9 to 7.1 they found that rate of hydrolysis to give ASP and development of turbidity are almost parallel, while at pH 9.3 rate of hydrolysis is faster than development of turbidity. Subsequently Oakley and Warrack(4) found that development of turbidity in lecithovitallin altered by lecithinase C was suppressed by high concentrations of human sera. MacFarlane (personal communication) observed that development of turbidity but not hydrolysis of lecithin by *Cl. welchii* toxin was inhibited by high concentrations of normal human, guinea pig and rabbit sera. By simple ammonium sulfate fractionation this author was able to demonstrate that the inhibitor was present only in the "albumin" fraction of sera. Brown *et al.*(13) have reported a coprotein in plasma which may act as an acceptor substance in conjunction with clearing factor. Both coprotein and L-FP are present in the supernate when sera are precipitated by 50% saturation with ammonium sulfate and neither is destroyed by dialysis. Both appear to prevent opalescence in sera in which lipids have been altered, coprotein working in conjunction with clearing factor and L-FP in conjunction with lecithinase C. Neither the effect of L-FP on lipids treated with clearing factor nor the effect of coprotein on lipoproteins acted on by lecithinase C has been investigated. Further work is necessary before the relation if any between L-FP and coprotein can be established.

The role of L-FP in suppressing development of opalescence in lipoproteins acted on by lecithinase C was not fully investigated. Although it was shown to suppress opalescence in human sera containing β lipoproteins its effect on β lipoprotein fractions was not studied. It appears possible that L-FP becomes bound to diglyceride when phosphorylcholine is liberated and that it takes the place of the charged ions of phosphorylcholine in making the lipoprotein soluble. Some evidence has been obtained that there is competition between L-FP and calcium and that the inhibitory effect of L-FP is blocked by high concentrations of calcium (unpublished).

Summary. 1. Hydrolysis of phospholipids of horse serum is not always accompanied by development of turbidity. 2. Although α_1 lipoprotein fractions from horse sera become opalescent with or without development of a precipitate following hydrolysis of the phospholipids by lecithinase C, cholesterol and the diglyceride portion of phospholipids remain bound to protein. 3. L-FP (a lipid-free protein present in human and horse sera) suppresses development of opalescence in α_1 lipoprotein fractions and whole human sera in which phospholipids are hydrolyzed by lecithinase C.

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Perphenazine (Trilafon®) and Chlorpromazine in Experimental Shock.* (24278)

F. E. Roth, J. M. McKenna,† and W. M. Govier
(With technical assistance of G. Crossin)
Department of Pharmacology, Schering Corp., Bloomfield, N. J., and Department of Biology, Seton Hall University, South Orange, N. J.

Reduction of mortality in animals subjected to hemorrhage has been classically associated with sympathectomy(1) or pretreatment with drugs(2,3,4) which prevent compensatory response of excessive and prolonged vasoconstriction. Bacterial, metabolic, and other factors have been implicated(5,6,7,8) in development of shock due to hemorrhage or trauma, but a causal relationship between these factors and shock is as yet incompletely established or controversial. It has been recently demonstrated that clinically useful ganglionic blocking agents possess protective action when administered prophylactically in experimental shock(9,10). The effectiveness of chlorpromazine(11,12,13,14,15,16) in similar studies may be at least partly attributable to its adrenergic blocking activity. Other possible mechanisms of protection, not involving autonomic blockade, have not been clarified at this time. In this report, the protective action of perphenazine (PPZ), a new phenothiazine derivative(17,18) was compared with that of chlorpromazine (CPZ) in hemorrhage shock in dogs and traumatic shock in rats.

Methods. Traumatic shock. Female Carworth rats (110-140 g) were subjected to tumbling trauma by the Noble-Collip procedure(19). The drum was revolved at 40 rpm for 600 revolutions. Drugs were administered intramuscularly 15 min before exposure to trauma. Animals which were dead on removal from the drum were not included in any calculations. The ratio of rats which had survived for more than 24 hours to those which were alive on removal from the drum was calculated for each treatment and dose group. Untreated control animals were used with each dose group and survival of these never exceeded 35%. *Hemorrhage shock* was produced in 73 healthy mongrel dogs (7-15 kg) anesthetized with sodium pentobarbital (30 mg/kg, iv). Bleeding was allowed from femoral artery to elevated reservoir bottle (2). The experiments were conducted during July, August, and September, in air-conditioned room, at 68-70°F. The entire procedure, including all equipment and operative technic, was performed under aseptic conditions. Blood pressure was recorded from a side-arm in the bleeding-line by mercury manometer. The ipsilateral femoral vein was isolated for direct drug administration. Immediately following vessel exposure all dogs received heparin (5 mg/kg, iv) and additional 30 mg injected into the reservoir bottle. Following arrangement of femoral arterial bleeding system, the animals were allowed to bleed into the maximally elevated reservoir bottle until the blood column height had equilibrated with the blood pressure. Two dogs were simultaneously prepared in this fashion, one receiving drug treatment (doses expressed in terms of free base) and the other acting as untreated control. Immediately after equilibration, a test drug was slowly administered, iv, and 15 min allowed for stabilization of blood pressure. The response to drug was a gradual decline in pressure not exceeding 15-30 mm Hg. During this drug-induced hypotensive phase there was a slight automatic reinfusion of blood back into the animal. Hemorrhage was then simultaneously initiated in both dogs by gradual lowering of reservoir bottles for 7-10 min to attain desired blood pressure level of 30 mm Hg. Constancy of

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® TRILAFON is trade name of Schering's perphenazine (1-(2-hydroxyethyl)-4-[3-(2-chloro-10-phenothiazinyl) propyl] piperazine dihydrochloride).

TABLE I. Effects of Perphenazine and Chlorpromazine on Survival of Rats Exposed to Traumatic Shock (Noble-Collip Drum).*

Drug	Dose, mg/kg (intramusc.)	Survival ratio, No. survivors/No. tested	
			%
Controls	—	32/103	31
PPZ	1.5	23/26	88
	.8	7/7	100
	.5	3/4	75
	.4	5/9	55
	.2	3/9	33
CPZ	1.5	22/26	85
	.8	9/9	100
	.4	8/8	100
	.2	9/12	75

* 600 rotations at 40 r.p.m.

this pressure level was self-regulatory(2). Initial bleeding volume (IBV) was recorded, and subsequent readings were made at 10 minute intervals, or less. The amount of blood which slowly accumulated subsequent to the IBV reading is the secondary bleeding volume (SBV). The IBV plus the SBV is maximal bleeding volume (MBV). When the compensatory action necessary to maintain the set blood pressure began to fail there was an automatic reinfusion of blood (ARV) back into the animal. Criteria for termination of experiment were: 1) The ARV having reached 25% of the MBV, or 2) survival of animal for 8 hours at hypotensive level of 30 mm Hg. It has been our experience that less severe criteria for termination of experiment were inadequate for demonstration of a uniformly high mortality rate in the control group. Termination proceedings were instituted by rapid raising of reservoir bottle so that all shed blood was returned to the animal, and all but approximately 6 inches of residual line-volume was slowly forced into the artery under air pressure. When stabilized, blood pressure was recorded for the last time, and usually ranged from 70-80 mm Hg. The femoral arterial cannula was removed, the artery ligated and the incision closed with skin clips after dusting with sulfadiazine powder. An animal was considered a survivor of hemorrhage shock if it was alive after 96 hours. Survivors or dogs which died during experiment demonstrated varying degrees of "typical shock" pathology, namely, intestinal and endocardial hemorrhage and liver engorgement.

Results. Statistical analysis of data in Tables I, II, and III demonstrated the following similarities or distinctions ($p<0.05$) between drug-treated and control groups:

Table I. Traumatic shock. Survival ratio of PPZ-treated rats was significantly greater than that of controls at doses between 0.5 and 1.5 mg/kg. The same protection was observed in the CPZ-treated groups, but CPZ was more effective than PPZ at doses below 0.5 mg/kg. It also was observed that CPZ had lesser "tranquilizing" potency than PPZ(17) since no sedation was apparent at doses of 0.4 to 0.2 mg/kg of CPZ; whereas, animals treated with 0.2 mg/kg of PPZ still showed some motor depression, not associated with the induced shock.

Table II. Hemorrhage shock. Survival ratio of PPZ-treated dogs (1 mg/kg) was significantly greater than that found for controls; whereas, the ratio for CPZ-treated dogs (1 mg/kg) was not. However, the ratio for the combined drug-treated groups (9/32) was significantly greater than that of controls. There was no significant difference in survival between CPZ and PPZ-treated groups. There was no difference in average MBV for either drug-treated or control groups. Average dur-

TABLE II. Effect of Perphenazine and Chlorpromazine on Bleeding Volume, Hypotension Time,* and Survival of Dogs Exposed to Hemorrhage Shock.

Drug, mg/kg (intrav.)	Avg max bleeding vol (ml/kg) ± S.E.		Avg hypotension time (min.) ± S.E.		Survival ratio, No. survivors/No. tested	
						%
Controls	58.1	1.3	134.9	16.3	1/41	2
PPZ (1.0)	57.2	2.1	248.1	31.3	6/16	37
CPZ (1.0)	53.1	2.1	281.6	32.0	3/16	19

* Duration of induced hypotension (30 mm Hg) before satisfying criteria for termination of experiment.

TABLE III. Effect of Perphenazine and Chlorpromazine on Bleeding Volumes at Various Stages of Exposure of Dogs to Hemorrhage Shock.

Drug, mg/kg (intrav.)	Avg bleeding vol (ml/kg ± S.E.)						Time to attain MBV (min. ± S.E.)	
	IBV		SBV		MBV			
Controls	45.4	1.2	12.8	1.1	58.1	1.3	39.6	3.8
PPZ (1.0)	33.6	1.9	22.9	1.8	57.2	2.1	89.2	13.5
CPZ (1.0)	33.1	1.9	19.9	1.8	53.1	2.1	83.6	12.7

ation of hypotension (maintained at 30 mm Hg) before criteria of shock were satisfied, was similar for drug-treated groups but significantly different from that found for control animals. This also was indicative of the protective action of the drugs. At a dose of 0.5 mg/kg, 1 out of 5 (20%) PPZ-treated dogs survived. At a dose of 3 mg/kg, 3 out of 7 (43%) CPZ-treated dogs survived. Although these studies are considered incomplete because of the few animals involved, it does indicate that significant protection can be achieved with CPZ.

Table III. The average IBV or SBV values were similar for both drug-treated groups but differed significantly from those values found for the controls. This also was true in regard to the time it took for the IBV to reach the MBV.

Discussion. It may be concluded that control dogs bled more initially (IBV) than did the treated dogs, and subsequently bled less than test dogs in the SBV period. Treated animals bled more slowly and steadily but no less *in toto* (MBV) than did the controls. This pattern of hemorrhage as to rate or volumes (IBV and SBV) in the drug-treated dogs may be attributed, for the most part, to prevention of rapid and severe vasoconstriction(2,3). It appeared that the adrenergic blocking action of PPZ or CPZ(18) was manifested to a moderate, but not marked, degree since the drug-treated dogs were able to bleed the same amount as did the control animals. Other, as yet undefined, mechanisms of the "anti-shock" action of these compounds also may be operating. However, at this time the evidence of a great increase in circulating epinephrine and norepinephrine following hemorrhage(20,21) appears to favor the concept of protection, by adrenergic blockade of excessive vasoconstriction and its pathologic sequelae. As has been reported for CPZ(22), the protective action of PPZ in preventing death following severe hemorrhage or trauma may be of clinical use in preventing untoward reactions to "stress situations."

Summary. 1) Pretreatment of rats with perphenazine or chlorpromazine (0.5 - 1.5 mg/kg, im) afforded significant protection against lethal effects of Noble-Collip drum trauma. Chlorpromazine was still effective at doses of 0.2 to 0.4 mg/kg, whereas perphenazine was not protective in this dose range. Pretreatment of dogs with perphenazine (1 mg/kg, iv) significantly protected against lethal effects of hemorrhage under aseptic conditions. Chlorpromazine did not display significant protection at 1 mg/kg, but it did appear to be effective at 3 mg/kg in a limited study. Both drug treated groups (1 mg/kg) bled the same maximal volume as did the controls but at much slower rates when systolic pressure was maintained at 30 mm Hg. 2) Reduction of mortality in pretreated animals subjected to experimental shock may be due, at least in part, to adrenergic blocking activity of the test compounds.

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Fibril Formation in Stationary Cultures of Mouse Lung Cells. (24279)

ROBERT W. PUMPER (Introduced by R. J. Winzler)
Department of Microbiology, University of Illinois College of Medicine, Chicago

It has recently been reported(1) that a strain 929 of L fibroblasts agitated in a serum-free medium gave rise to the formation of fibers. This fiber material was shown to be digested by collagenase, partially degraded by trypsin, and resistant to alpha amylase and hyaluronidase. The observations reported here are cited to bear out this report using a different cell line in a non-agitated medium, to present new information regarding the period of appearance and disappearance of the fibrils, and to present photographic evidence of the cell fibril complex.

Materials and methods. The line of cells used in this report was isolated from lung tissue of new-born Swiss mice, NIH strain. The original isolation was made using trypsin dispersed cultures prepared by a procedure similar to that of Youngner(2). Prior to their growth on a serum-free medium the cells were in their 110th passage on a medium consisting of 10% horse serum and 90% medium #199 (3). Penicillin and streptomycin were added in a final concentration of 50 units each per ml. Morphologically the cells appeared fibroblast-like, although small numbers of epithelioid cells were in evidence. The cells were routinely passed every 4 days by trypsinizing, centrifuging, resuspending, and inoculating, (hemocytometer count) 3 x 10^5 cells per ml in T-30 flasks, the total volume being 5 ml.

During the course of adapting these cells to a serum-free medium consisting of 99% medium #199, 1% Difco Bactopeptone, and 100 mg% glucose (BPD) it was noticed that a floating layer of cells was formed. These cells were separate from the cell layer which had attached to the glass surface, and appeared in the early passages after 5 to 8 days of incubation at 37°C. The floating cells were seen as discrete patches often 3-4 mm in diameter. Photomicrographs of this material were obtained by inverting and reinverting the T-30 flasks so that the unattached cells adhered temporarily to the glass surface opposite the normal cell layer. In this way the natural configuration of the material could be studied. Portions of the material were then removed and tested with several enzymatic preparations. Results of these tests showed that the material was resistant to digestion by hyaluronidase and trypsin but susceptible to digestion with collagenase.

Fig. 1 shows a typical growth of mouse lung cells on a glass surface using a 10% horse serum medium. The cells form an even sheet which is extremely difficult to dislodge physically. Fig. 2 illustrates the tangled mass of

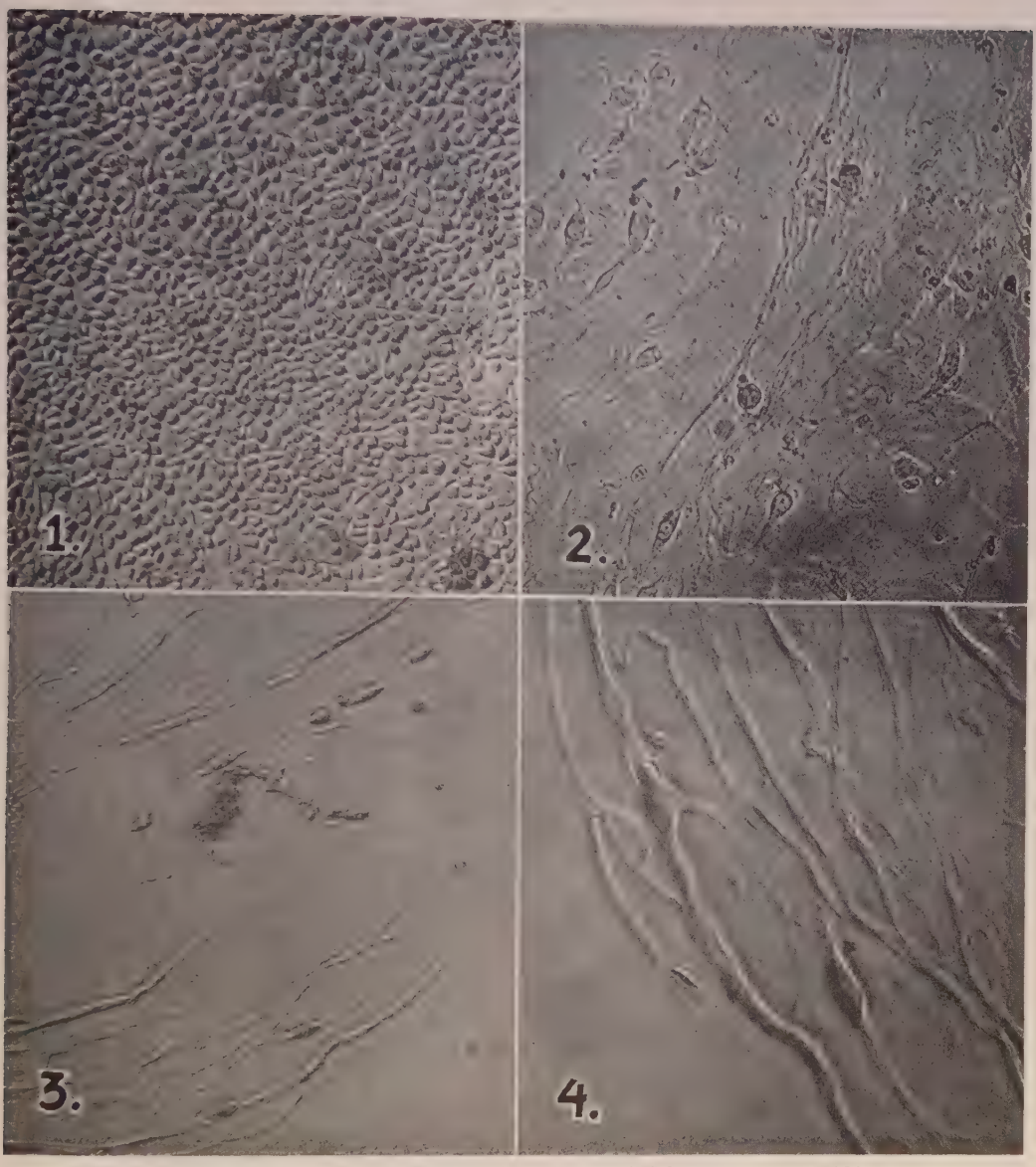

FIG. 1. Normal culture of mouse lung cells grown on 10% horse serum. × 180.

FIG. 2. Floating sheet of cells and fibers seen on 1st passage of mouse lung cells on Bactopeptone-dextrose medium. × 180.

FIG. 3. Discrete fiber material shown in association with mouse lung cells, 5th passage on Bactopeptone-dextrose medium. × 180.

FIG. 4. Higher magnification of the material shown in Fig. 3. × 400.

material which made up the floating layer in the first passage on BPD medium. This response gave way to the more discrete fiber formation seen in Fig. 3. Notice that the fibril material seems to arise from single cells and crosses the entire field of vision. This type of response did not occur until the 4th to 6th passage, Fig. 4 is an enlargement of the material shown in Fig. 3 and affords a better view of the origin of the fibers. The transition from large masses of floating material to better defined strands was not an immediate but a gradual one.

These cells are now in their 21st passage on

BPD and no longer give rise to fibril formation upon routine passage. It is possible to induce a surface type growth by scraping the cells from the glass surface and transplanting them into new BPD media with a minimum of agitation. Under these conditions the floating cells never produce the long strands shown in Fig. 3, but grow and expand as cell sheets with a morphology similar to, but not as compact as, the attached cell sheet.

Early passages of the L cell line(4) in the BPD medium produce floating cell sheets similar to those seen with the mouse lung line. The discrete fibrils seen in the case of the mouse lung cells have not been seen with the L cells.

Discussion. As a result of the above investigation certain facts concerning fibril formation can be stated. It is certain that fibrils can arise without agitation of the medium, and are therefore not the result of entwining of single strands of material. From the photomicrographs it appears that the fibrils arise from the cell body *per se* and are not the result of entrapment of cells in fibrous material released by the degenerated cells. The fact that the fibrils are no longer seen once the cells become adapted to a rapid growth in the BPD medium points up the fact that they may be the result of unfavorable growth conditions as previously hypothesized(1). Attempts to stimulate a similar response by other sub-optimal conditions in the absence of BPD have failed. It is probable therefore that the Bactopeptone itself is at least partly responsible for formation of this material.

Summary. The presence of fibril-like material in a strain of mouse lung cells grown in a serum-free medium is described. This material was resistant to digestion by hyaluronidase and trypsin and susceptible to digestion with collagenase. The period of appearance and disappearance is discussed and photographic evidence of the cell body-fibril association is presented.

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Elastin Content of the Human Lung.*†‡ (24280)

A. M. BRISCOE AND W. E. LORING

Departments of Biochemistry and Medicine, Columbia University College of Physicians and Surgeons, and Chest Service, Bellevue Hospital; and the Department of Pathology, New York University College of Medicine, New York City

Elastic tissue is readily demonstrated histologically in the parenchymal tissue of the human lung but no quantitative chemical studies of it have been reported in this tissue. Such investigations are now in progress in this laboratory. Chemical estimation of elastic tissue at present must be based on its content of the fibrous protein, elastin. The purpose of this communication is to report the amount of elastin in the human lung, and the correlation between pulmonary elastin concentration and age. In addition, the normal concentrations of elastin have been compared with those obtained in lung parenchyma subjacent to areas of bulla formation. It has been suggested that elastic tissue plays a role in the pathogenesis of such lesions.

Of several methods of elastin determination which have been reported(1,2,3), the method of Lowry *et al.* was chosen for study of the

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lung, and adapted to exclude insoluble substances other than elastin which may be encountered in lung tissue.

Materials and methods. The lungs examined were fresh specimens obtained at the time of autopsy. Causes of death were either traumatic or of extrapulmonary nature. Tissue was selected which was ostensibly free of pulmonary disease.

Elastin was determined by the following modification of Lowry's method(1). The samples were minced, ground to a paste, suspended in 0.1N NaOH and digested in a boiling water bath for 10 minutes. The insoluble material was separated by centrifugation, resuspended in NaOH and digested similarly twice more. The residue was then washed, dried and weighed. It consisted of elastin and insoluble foreign material and was designated W-1. Elastin was then removed by autoclaving in 6% KOH for 45 minutes. The residue was again washed, dried and weighed to give W-2. Difference in weight between W-1 and W-2 was assumed to be the weight of elastin in the sample. The amount of soot and other insoluble foreign matter in the lung could also be calculated from the difference between W-2 and the weight of the empty tube.

For each lung, 4 contiguous pieces weighing between 1.0 and 1.7 g were selected for quadruplicate analyses. These specimens were taken from the left upper lobe in nearly all cases. Pleura and large blood vessels and bronchi were excluded. Water content was determined in other specimens from the same area. In addition, selected specimens were taken for routine histological examination using Weigert's resorcin-fuchsin elastica stain and Golder's modification of Masson's trichrome stain, respectively. In 4 lungs with bullae, specimens for analysis were taken from the tissue directly beneath the bullae. The results are reported as per cent of dry weight of the tissue.

Results. Analyses of normal pulmonary parenchyma from men and women between 16 and 93 years of age revealed that elastin constituted an average of 8.28 ± 0.6% of the solids of the lung. The range of values was from 1.75 to 13.1%. These results are summarized in Table I.

TABLE I. Concentration of Elastin in the Human Lung.

Group	No.	Elastin, % dry wt
Normal men	17	8.08 ± 1.4
" women	12	9.48 ± 1.2
Men with bullae	4	4.61 ± .9

TABLE II. Comparison of Concentrations of Insoluble Residue and Elastin in Lung.

Age	Insol. residue, % dry wt	Elastin, % dry wt
25	.1	7.23
22	1.65	3.65
42	1.53	11.2
65	4.70	11.5
60	18.1	7.5
66	12.6	11.2

The last step in the revised method of analysis described above was added to correct for insoluble residue in the lung. Data calculated from 6 experiments are presented in Table II to illustrate the necessity for this correction. The accumulation of this residue increases with age and is quite evident on gross inspection of the lungs.

In Fig. 1, results of analyses of lungs from 29 apparently normal men and women are plotted against age at time of death. Data

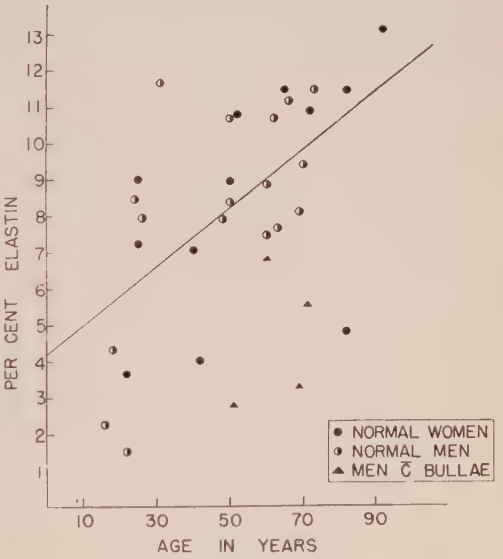

FIG. 1. Elastin concentration plotted against age: % of dry wt of tissue.

obtained from tissue under bullae in lungs of 4 older men are also included. Statistical analysis of this graph shows a highly significant increase in relative amount of elastin with age ($t = 3.87$, $p > 0.001$). The regression slope shows an increase in elastin content of the lung of 0.816% per decade of life. On superficial examination of the graph, women over 50 appeared to have more pulmonary elastin than men in this age group. However, when subjected to statistical analysis, the difference in this small series was found to be insignificant ($t = 0.75$, $p = 0.4$). Comparison of the elastin content of sub-bullous tissue with that of normal lung parenchyma of older men revealed a highly significant decrease in the former ($t = 5.09$, $p > 0.001$).

Histological studies of the elastin isolated by our method showed that the end-product was composed only of elastic tissue. However, one preparation contained traces of collagen deep within the walls of a pulmonary arteriole.

Discussion. It has been generally believed that the elastic tissue content of the aging lung is reduced. Our measurements must be viewed critically and are simply a prelude to further investigation. Although the method was well standardized and corrected for errors due to foreign material in the lung, results obtained from lungs of different ages may not be entirely comparable. Pulmonary elastin may change physically and chemically with age as has been demonstrated in elastin from the aorta(4), and these changes may affect the efficiency of isolation and separation. Confirmatory studies of elastin content by other methods are needed. Simultaneous measurement of pulmonary collagen is contemplated in view of the close relationship between collagen and elastin. It is of considerable interest that concentration of elastin in the lung is relatively high. Of the tissues thus far analyzed, only the aorta and the ligamentum nuchae contain more elastin than the lung(1,2).

Summary. 1. An alkaline digestion method for quantitative determination of elastin has been adapted for study of lung parenchyma. 2. Concentration of elastin in the adult human lung has been determined over a wide age span, and has been found to increase with age. 3. Concentration of elastin in lung parenchyma subjacent to areas of bulla formation is significantly reduced.

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Effect of 6-Mercaptopurine on Antibody Production.* (24281)

ROBERT SCHWARTZ, JANICE STACK AND WILLIAM DAMESHEK
Blood Research Laboratory, New England Center Hospital, and the Department of Medicine, Tufts University School of Medicine

Since immunologic mechanisms participate in the rejection of homografts by experimental animals(1), some modification of the immune response is necessary in order to achieve a "take" of the grafted tissue. Alteration of antibody production may occur naturally, as in agammaglobulinemia(2), or may be induced by means of X-radiation(3) or cortisone(4). Protein(5) or vitamin(6) deficiencies obtained by means of special diets (7) or by the action of amino acid analogues (8) may lead to suppression of antibody formation and to prolonged survival of skin homografts(9). In the special case of tumor

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homografts, treatment of the recipient with cortisone may result in a "take"(10). Since bone marrow transplantation has been suggested as a possible treatment for radiation injury, aplastic anemia and some neoplastic diseases(11), our laboratory has become interested in altering antibody formation by various means. The purpose of this paper is to present data which indicate that an antagonist of nucleic acid metabolism, 6-mercaptopurine (6-MP), can abolish the antibody response to a purified antigen.

Method. White New Zealand strain rabbits weighing about 3 kg and maintained on a stock *ad libitum* diet were used. All animals were injected intramuscularly with 50 mg of crystalline bovine albumin (Armour) 3 times weekly for 2 weeks. The first day when antigen was injected was counted as day 0; time prior to this was designated by a (−) sign and time subsequent to day 0 was designated by a (+) sign. The experimental animals were given 6-MP, 3 mg/kg/day according to the following schedule; Group 2M (10 animals): from days 0 to +11; Group 3M (5 animals): from days 0 to +21; Group 4M (5 animals): from days −7 to +4; Group 5M (5 animals): from days +7 to +18; Group 6M (5 animals): from days +18 to +29. There were 10 animals in the control group. Bleedings were made from a marginal ear vein at the times indicated by Fig. 1. Antibody was measured by the tannic acid hemagglutination technic of Stavitsky(12). Results are expressed as the $\log_2$ of the highest serum dilution which gave a +1 pattern.

Results. The course of the experiment is depicted graphically in Fig. 1. Low titers of antibody could be detected in the control group by day +4, with the peak titer at about day +16; thereafter a plateau persisted through the last bleeding at day +38. No antibody could be detected in Group 2M, given 6-MP from days 0 to +11 until 7 days after the antimetabolite had been discontinued; a peak titer of antibody was reached 14 days after this time.

Group 3M, given 6-MP from days 0 to +21, made no detectable antibody during the 38 days of the study. Clinical evidence of toxicity was apparent by day +14, and was manifested by weight loss, poor appearance of fur and diarrhea. One animal died during the study on day 20. Two animals suffered spontaneous abortion. All animals of this group were neutropenic by day 14.

Group 4M, given 6-MP from days −7 to +4 had no detectable antibody on day +4, but by day +14 antibody production was nearly equal to the control values. Group 5M, which received 6-MP from days +7 to +18 showed an apparent cessation of antibody production between days +4 and +14. Four days after the last injection of 6-MP, however, antibody could again be detected and thereafter the titers rapidly approached the control levels. Administration of 6-MP during the height of antibody response, as in Group 6M, had no effect on antibody production.

Discussion. The data reported herein demonstrate that 6-MP, a powerful nucleic acid antimetabolite, abolishes the rabbit's immune response to bovine serum albumin when given simultaneously with the antigen. Although the site of action of 6-MP with respect to antibody formation is unknown, it seems unlikely that suppression of protein formation is an important factor, since administration of the drug during the height of the immune response had no effect on the titer of antibody in the serum. It may be inferred from the data that 6-MP had no effect on the anamnestic response, but did have a pronounced effect on the primary response. It is probable that 6-MP interferes with the utilization of purines for nucleic acid synthesis(13) and, if antibody producing tissue can be assumed to be in a hypermetabolic state, then such interference could have a profound and relatively selective action on the immune response. The presumed organization of "templates" for antibody formation is intimately concerned with the nucleic acid metabolism of the cell (14), and it is possible that 6-MP disrupts either the "information center" (DNA) or the actual template (RNA-protein) of antibody forming cells, thus leading to depression of the primary response. Studies to determine the mechanism and site of action of 6-MP in

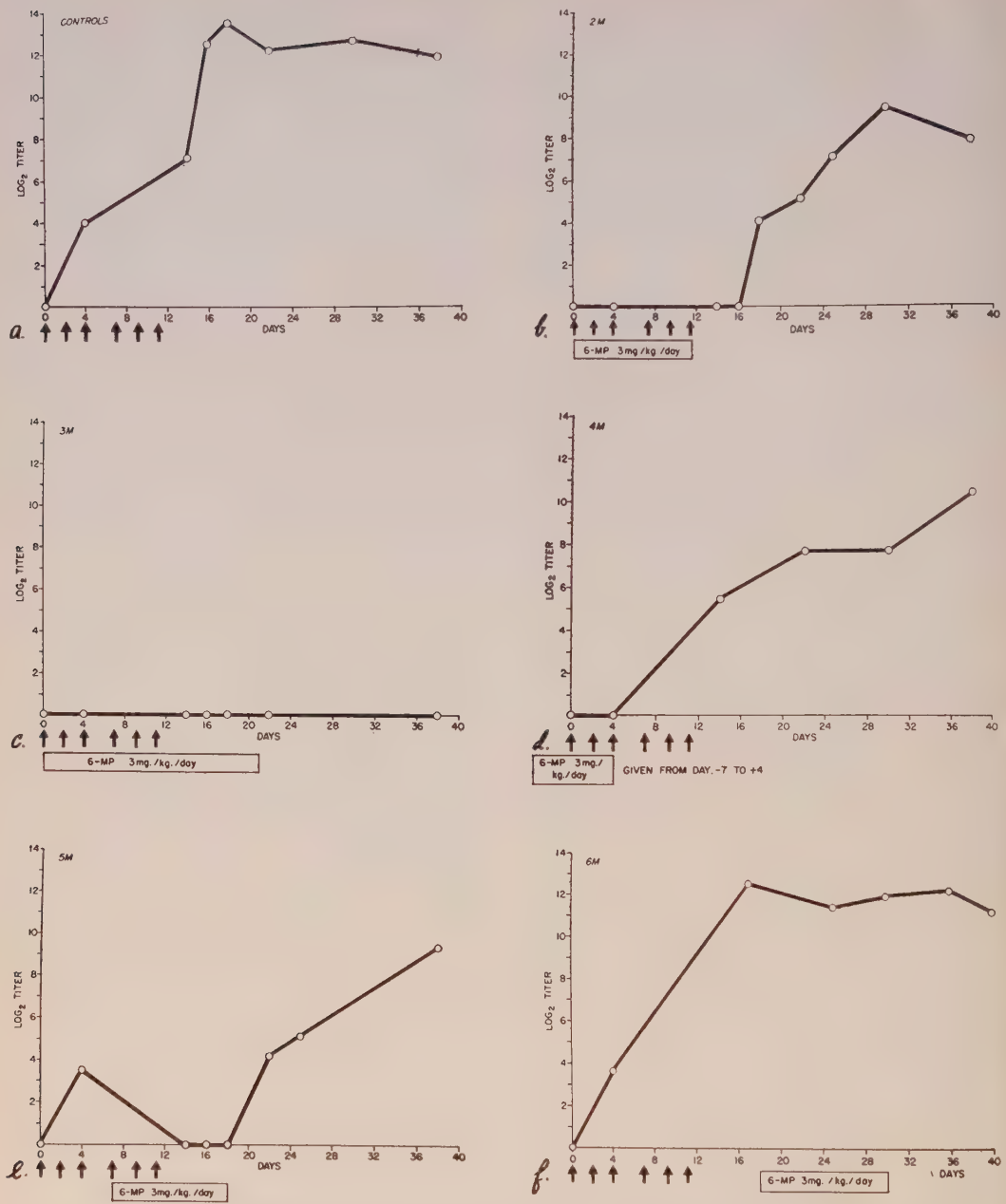

FIG. 1. Course of immune response in control and 6-MP treated rabbits. Each vertical arrow represents antigen inj. Time of administration of 6-MP is represented by the clear block. Each value depicted in the graph represents avg titer for the group.

regard to antibody formation, as well as its effects on the immune response to cellular antigens, are in progress.

Summary. 1. The effect of 6-MP on antibody response of hyperimmunized rabbits has been studied. 2. When given simultaneously with the antigen, 6-MP profoundly suppresses the formation of humoral antibody. When given prior to antigenic stimulation, the effect of 6-MP on antibody production is slight. There is no effect on amount of antibody in the serum when 6-MP is given during the

height of antibody production. 3. It has been tentatively concluded that the action 6-MP is on the primary antibody response, rather than on the anamnestic response.

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Sensitivity of Mice to Endotoxin after Vaccination with BCG (*Bacillus Calmette-Guérin*)* (24282)

EMANUEL SUTER,† GRACE E. ULLMAN AND ROBERT G. HOFFMAN

Department of Microbiology, College of Medicine, and Statistical Laboratory, University of Florida, Gainesville

The establishment of tolerance to endotoxins derived from gram negative bacteria upon repeated injection is attributed to increased activity of the reticulo-endothelial system (RES). Tolerance is effective against many manifestations of endotoxin activity, such as fever, leukopenia, shock, and Shwartzman reactions(1) and may last in mice for 4 months(2). Single or repeated injections of endotoxin result in increased clearance of particles or large molecules from the blood stream by the RES(3). Similarly, infection with virulent tubercle bacilli or vaccination with BCG were found to result in increased clearance by the RES(4). In view of these findings it appeared possible that BCG vaccination might induce tolerance to endotoxin. This view is supported by the fact that a single injection of endotoxin increases resistance to tuberculous infection(5) indicating some relationship of the host's reaction to these 2 biologically active agents. Experiments were therefore undertaken to study the reaction of mice infected with tubercle bacilli to endotoxin derived from gram negative bacteria.

Methods and materials. Swiss albino mice of the Webster strain were used. Lipopolysaccharide was prepared according to the technic described by Westphal and Lüderitz from a strain of *E. coli B*(6). Preparations were also obtained from the Difco Laboratories, Detroit, Mich. (Lipopolysaccharide *E. coli* 026:B6) and from Wander S. A., Berne, Switzerland (Pyrexal Wander).‡ The lipopolysaccharide was suspended in physiological solution of sodium chloride and was injected intraperitoneally. For vaccination 0.2 ml of a BCG§ culture grown for 7 to 10 days in the liquid tween-albumin medium was injected intravenously. In some experiments cord factor derived from virulent tubercle bacilli‖ was

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§ BCG was obtained from the Henry Phipps Inst., Philadelphia, Pa.

‖ Cord factor was kindly supplied by Dr. Hubert Bloch, Dept. of Microbiology, Univ. of Pittsburgh Medical School, Pittsburgh, Pa.

used for the preparatory injection. Cord factor was dissolved in mineral oil (25 μg/ml) and 0.2 ml (*i.e.* 5 μg) was injected intraperitoneally twice at a 48-hour interval. In each experiment LD_{50}'s were determined simultaneously in control and experimental animals by injecting intraperitoneally groups of 3 to 5 mice each with dilutions of lipopolysaccharide containing 1,000, 100, 10 and 1 μg per dose (0.5 ml) respectively. Deaths were recorded up to 72 hours. All animals were usually tested 7 to 10 days after the preparatory injection with BCG or cord factor.

For evaluation of the results the square root of dosage units was used, because the square root transformation yielded a closer approximation to a straight line upon probit transformation of the ordinate, than did the logarithmic transformation. A probit equation using the maximum likelihood method was then fitted to the groups to obtain an LD_{50} and a standard deviation(7).

Results. The results are summarized in Fig. 1, in which each bar represents the square root of the LD_{50} in μg, the bracket indicating 2 standard deviation limits. 1) *Effect of vaccination with BCG on sensitivity to endotoxin*: (Chart A) The LD_{50} for endotoxin was 357 μg in control mice and 7 μg in mice which had been vaccinated 7 to 10 days previously. The course of the shock induced by endotoxin in vaccinated animals appeared to be more acute than in controls, *i.e.* death usually occurred within 6 to 10 hours in the vaccinated group and within 12 to 48 hours in the controls. When mice were tested with endotoxin at various intervals of time after vaccination, some increase of susceptibility to endotoxin became manifest within one to 3 days after injection of BCG. A rather sudden change of LD_{50} appeared between the 5th and 7th day after vaccination (Fig. 1, chart A). The state of hyperreactivity was still detectable 70 days after vaccination. 2) *Effect of cord factor derived from tubercle bacilli on sensitivity to endotoxin.* In an attempt to investigate whether any single component of tubercle bacilli could induce a similar state of hyperreactivity to endotoxin, the cord factor, isolated by Bloch from tubercle

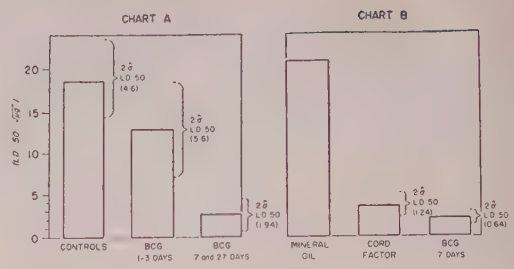

FIG. 1. Mean of square roots of LD_{50} of endotoxin in μg in mice pretreated with BCG, cord factor or mineral oil. The brackets indicate 2 standard deviation limits. *Chart A:* Comparison of LD_{50} in control mice with that in mice vaccinated with BCG 1 to 3 and 7 to 27 days previously. *Chart B:* Comparison of effect of pretreatment with cord factor, BCG and mineral oil.

bacilli was selected. This component, a trehalose dimycolate, is known to cause death of mice after repeated injections of small doses (8). Cord factor dissolved in mineral oil was injected twice and the mice were challenged with endotoxin 7 days later. The LD_{50}'s were as follows (Fig. 1, chart B): In mice which had received cord factor 15 μg, in controls injected with mineral oil 441 μg and in BCG vaccinated animals 6 μg.

Discussion. Contrary to expectation, the results clearly indicate that mice infected with an attenuated strain of tubercle bacilli, namely BCG, became highly sensitive to lethal doses of lipopolysaccharide derived from gram negative bacteria. This hyperreactivity appeared within 5 to 7 days and lasted for at least 70 days. In preliminary experiments in which mice were infected with a virulent strain of tubercle bacilli, a similar degree of hyperreactivity to endotoxin was found. Compared with the effect of BCG vaccination, no correlation existed between severity of infection and degree of hyperreactivity to endotoxin. It is known from earlier experiments that certain acute and subacute bacterial infections induce a state of increased sensitivity to bacterial endotoxins. That is, a single injection of bacterial culture filtrate or preparations of endotoxin causes a generalized Shwartzman reaction in rabbits or guinea pigs which have an active infection with cholera bacilli, streptococci or *Coxiella burneti*(9). In these experiments, the animals were tested one or 2 days after infection. When guinea

pigs were injected with a large dose of tubercle bacilli (5 to 10 mg) and challenged intraperitoneally with either suspensions of heat killed *E. coli* or with culture filtrates, the animals showed a much more severe response than control animals leading to shock and death(10). The high degree of reactivity to these materials was interpreted as depending upon the persistence of nodular lesions within the omentum due to deposit of large masses of BCG. The mechanism of this hyperreactivity is unknown and usually described as a generalized Shwartzman phenomenon. Sufficient data are not available from our experiments to decide whether this high degree of sensitivity coincides with or is dependent upon actual multiplication of tubercle bacilli. It had been shown that BCG multiplies in tissues of mice for approximately 2 to 3 weeks, and that the population of bacilli declines gradually thereafter(11). In our experiments the state of hyperreactivity was demonstrable beyond this time, and probably not related to continued growth of bacilli in the tissues.

The fact that cord factor, a trehalose dimycolate derived from virulent tubercle bacilli, induced a similar state of hyperreactivity as did BCG, would indicate that this reactivity was not related to delayed hypersensitivity to tuberculin, because cord factor does not induce tuberculin hypersensitivity of this type. Recently it has been shown that tuberculin shock in guinea pigs may be due to a generalized Shwartzman reaction rather than to an immunological reaction of the delayed type. These conclusions were based on the fact that tuberculin sensitive guinea pigs resisted high doses of tuberculin when they were rendered tolerant by repeated injections of lipopolysaccharide from *E. coli*(12). Experiments are in progress to investigate the mechanism of the phenomenon here described and to explore its possible role in pathogenesis of the tuberculous infection.

Summary. 1. It is shown that a single intravenous injection of BCG in mice induces a state of hyperreactivity to bacterial endotoxin. The LD_{50} for *E. coli* lipopolysaccharide is decreased from 357 μg in controls to 6 or 7 μg in BCG vaccinated animals. This change appears around the 5th day after vaccination. 2. Trehalose dimycolate, a component derived from virulent tubercle bacilli induces a similar state of hyperreactivity to endotoxin upon single or repeated injections.

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Osmotic Resistance and Post-Transfusion Survival of Human Erythrocytes Stored in the Presence of α-Tocopheryl Disodium Phosphate. (24283)

Irwin M. Weinstein, James C. Mathies, Richard Katzman and F. Patricia Forney (Introduced by M. H. Maxwell)

Wadsworth Hospital, Veterans Administration Center, Los Angeles, Calif.; the Department of Medicine, University of Chicago; and the Chemistry Department, Baxter Laboratories, Inc., Chicago, Ill.

Human erythrocytes undergo a progressive decrease in osmotic resistance during storage at 4° and this effect has been employed as an *in vitro* measure of the state of preservation of blood(1-2). During the course of a screening program for new blood preservatives it was observed that tocopheryl phosphate salts, over a narrow concentration range, markedly retarded this decrease in osmotic resistance. The effect of tocopheryl phosphate salts on post-transfusion survival of red cells is here described.

Methods. Osmotic resistance was measured at 0.6% buffered saline by the procedure of Parpart *et al.*(1.) Manner of preparation and handling of samples was essentially that of Schales(2). Phosphate partitions were determined on whole blood by the method of Umbreit, Burris, and Stauffer(3).

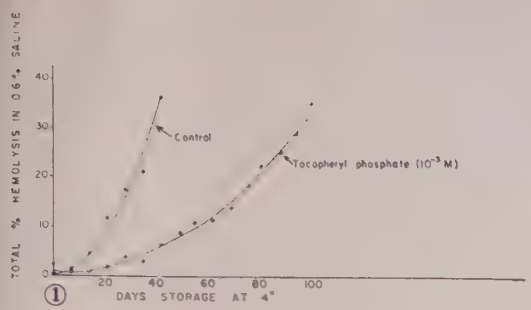
Post-transfusion survival was determined in normal human volunteers using Cr^{51} labeled red cells as reported previously(4). One hundred ml of blood was withdrawn by gravity from each subject into sterile bottles containing either anticoagulant acid citrate solution B. USP XV (ACD) alone or ACD plus disodium α-tocopheryl phosphate to a final molarity of 10^{-3}. The blood was then refrigerated at a constant temperature of 4°C for varying periods of time. Prior to reinjection the blood was labeled with 150 μc of Cr^{51}, allowed to stand at room temperature for one hour, then the red cells were washed 3 times with cold physiologic saline. Fifty ml of the washed, Cr^{51}-labeled erythrocytes were then injected into the same donors. Counts/min/ml of red blood cells at 15 min after injection were used as 100% activity. Samples of blood were withdrawn at appropriate intervals beginning 24 hours after injection. Survival was calculated by the formula.

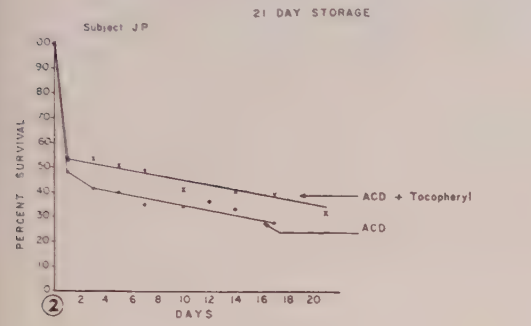
$$\% \text{ survival} = \frac{\text{c./min./ml sample x 100}}{\text{c./min./ml 100\% sample}}.$$

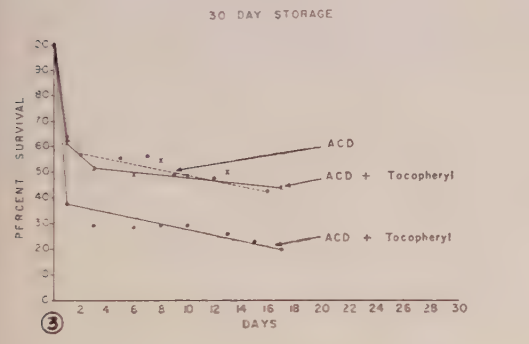
Results. The effect of storing ACD blood in the presence of 10^{-3}M disodium α-tocopheryl phosphate is demonstrated in Fig. 1. The total % hemolysis at 0.6% buffered saline after 100 days of storage was slightly less than that obtained in the controls after 42 days of storage, indicating marked "protection." The effect of varying the concentration of tocopheryl phosphate was also determined (Table I). Concentrations of 2 x 10^{-3}M were quite hemolytic, while concentrations of 10^{-4}M and less had no detectable effect. The "protective effect" was obtained at a level just below that inducing extensive hemolysis. Preliminary results with the sodium salt of tocopheryl succinate (Table I) gave results comparable to those obtained with the phosphate ester indicating the general nature of the effect.

Addition of tocopheryl phosphate to the solutions employed in the osmotic resistance test at concentrations comparable to those obtained above, were without significant effect. Therefore, storage of ACD-blood in the presence of tocopheryl ester salts is required to obtain this effect on osmotic resistance.

It then became desirable to determine degree of preservation by actual post-transfusion measurements. Since α-tocopheryl phosphate is known to have anti-thrombic activity (5-6), it was first necessary to determine the effect of infusions of this compound on the coagulation system. Intravenous injection of 2.5 mg of disodium α-tocopheryl phosphate/kg body weight in the dog (rapid infusion), and 1.25 mg/kg body weight in man (slow infusion in 5% dextrose over 25 min) caused no significant alteration in bleeding time, coagulation time, platelet count, prothrombin time,







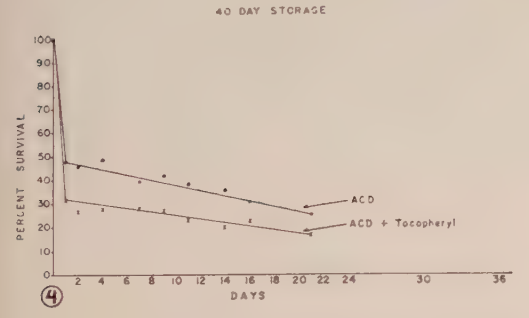


FIG. 1. Effect on osmotic resistance of storing human ACD blood at 4°C in presence of 10^{-3}M disodium dl-α-tocopheryl phosphate.

FIG. 2. Post-transfusion survival of human erythrocytes stored 21 days at 4°C.

FIG. 3. Post-transfusion survival of human erythrocytes stored 30 days at 4°C.

FIG. 4. Post-transfusion survival of human erythrocytes stored 40 days at 4°C.

thrombin coagulation time or recalcification time. Dog blood was then stored in ACD plus α-tocopheryl phosphate for 21 and 42 days at 4°, and reinfused into the donor dogs without toxic effects.*

Results of post-transfusion survival studies in humans† are presented in Fig. 2, 3, and 4. It is obvious that no enhancement of erythrocyte survival occurs when ACD-blood is stored in the presence of α-tocopheryl phosphate. Phosphate partition determinations (Table II), completed a few days before infusion, demonstrate that the energy-rich organic phosphate ester stores were equally depleted in the presence and absence of α-tocopheryl phosphate.

Discussion. The effects obtained with tocopheryl esters appear to parallel those obtained by Chaplin, Crawford, Cutbush, and Mollison(7) with a phenothiazine derivative (R.P. 3300) related to Phenergan. Both (a) reduce rate of decrease in osmotic resistance of stored blood; (b) are hemolytic at concentrations only slightly higher than required to give "protection"; (c) are surface-active in nature(7-8); (d) do not improve post-transfusion survival; (e) exhibit enhanced lytic effect on removal of serum proteins. Addition of 10^{-3}M α-tocopheryl phosphate to sheep blood did not cause hemolysis while addition to washed cells suspended in saline caused rapid, extensive, hemolysis. Therefore, the increased osmotic resistance of erythrocytes from blood stored in the presence of α-tocopheryl ester salts may be another example of the anomalous behavior of erythrocytes in the presence of sub-hemolytic amounts of hemolytic compounds.

Summary. Presence of 10^{-3}M disodium α-tocopheryl phosphate in human ACD-blood greatly retards rate of decrease in osmotic resistance of the erythrocytes that occurs on

* In this species the use of 10^{-3}M α-tocopheryl phosphate was detrimental as measured by spontaneous hemolysis and osmotic resistance.

† The clotting mechanism of these subjects was tested before and 15 min. after injection of the blood. No changes in bleeding, clotting, recalcification, prothrombin, prothrombin consumption, or thrombin times were noted.

TABLE I. Effects of Addition of Tocopheryl Ester Salts to Human ACD Blood on Erythrocyte Osmotic Resistance during Storage.

Compound added	Additive final molarity	% buffered NaCl in hemolysis test	Days blood stored at 4°C: 13	24	36	45	58	69	84	99
			% hemolysis							
Disodium d,l-α-tocopheryl phosphate	2×10^{-3}	.6	10.7	24.6	40.4					
	"	1.0	8.5	16.5	32.3					
	10^{-3}	.6	3.5	3.8	5.6	7.6	11.3	16.1	18.2	26.4
	"	1.0	1.9	2.8	3.4	4.3	5.1	6.0	9.0	12.6
	10^{-4}	.6	7.7	7.8	14.3	12.1	39.8			
	"	1.0	1.0	1.9	3.9	3.8	5.2			
d-α-tocopheryl succinate	10^{-3}	.6	1.5	3.8	4.3	4.9	6.9	8.8	10.2	15.7
	"	1.0	1.7	1.7	4.3	4.4	3.7	4.9	8.5	10.2
	None	.6	3.3	5.3	10.0	16.6	33.3	52.1		
	"	1.0	1.5	2.0	2.8	4.3	4.7	9.3		

TABLE II. Changes in Phosphate Partition of ACD Blood* on Storage at 4°C in Presence of 10^{-3}M α-Tocopheryl Phosphate.

Days blood stored at 4°	No. of subjects	Additive	% of total phosphorus: Inorganic	Easily hydrolyzable	Difficulty hydrolyzable	Non-hydrolyzable
0	8	None	11.4	18.4	10.0	60.2
18	2	Tocopheryl	74.4	9.5	4.8	11.3
24	2	"	77.7	6.9	4.3	11.2
24	1	None	74.1	8.9	4.5	12.5
36	1	Tocopheryl	83.4	4.0	2.5	10.1
36	1	None	77.9	7.7	4.0	10.4

* These human bloods were subsequently employed in post-transfusion survival experiments reported in Fig. 2, 3, and 4.

storage at 4°C. Post-transfusion survival of erythrocytes from blood stored in this manner is no better than that of erythrocytes from blood stored in ACD alone. It is emphasized that enhanced osmotic resistance of preserved blood is not necessarily related to enhanced post-transfusion survival.

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Chromium^{51} Elution from Hemoglobin and Intact Erythrocytes of Adults, Infants and Patients with Cooley's Anemia.* (24284)

Marion E. Erlandson, Irving Schulman, Beatrice Walden and Carl H. Smith
Department of Pediatrics, The New York Hospital-Cornell Medical Center

Stimulated by the discrepancy in values for life span of infant erythrocytes when measured by the Ashby and chromium^{51} technics (1), Suderman *et al.* recently demonstrated an accelerated rate of chromium^{51} elution in cord-hemoglobin solutions when compared with adult hemoglobin solutions(2). If the rapid elution rates found in cord-hemoglobin solutions are attributable to the presence of large amounts of fetal hemoglobin, similarly rapid elution rates would be expected in hemoglobin specimens from patients with homozygous Cooley's anemia where high percentages of fetal hemoglobin are found. If excessive elution also occurs from intact red blood cells in such patients, previous estimates of erythrocyte longevity in patients with Cooley's anemia might have to be invalidated when based on the chromium^{51} technic. Therefore, experiments were designed to compare chromium^{51} elution rates from intact erythrocytes and hemoglobin solutions from normal adults, infants, and patients with Cooley's anemia.

Methods. Venous blood was collected in acid-citrate-dextrose solution using 2 ml ACD per 10 ml blood. In the preparation of hemoglobin solutions, chromium^{51} as sodium chromate was introduced immediately into the blood-anticoagulant mixtures which were then incubated for 90 minutes at room temperature. Three microcuries of chromium^{51} were added for each ml of blood. (The amount of isotope employed was the amount routinely used in this laboratory for injection specimens in *in vivo* erythrocyte survival studies.) The tagged red blood cells were then separated by centrifugation, washed 3 times with isotonic sodium chloride, and hemolyzed by addition of water. The stroma was removed by agitation with toluene and subsequent centrifugation and filtration. The clear filtrates of hemoglobin solution were adjusted to approximately 12 g per 100 ml water. Counting specimens and dialysis specimens were prepared as described for the labeled erythrocytes.

In the preparation of intact erythrocyte suspensions, the cells were separated from the plasma-ACD mixture by centrifugation and resuspended in that amount of isotonic saline necessary to adjust the hemoglobin concentration to approximately 12 g per 100 ml of suspension. The plasma was saved. The saline suspensions of red blood cells were tagged with 3 microcuries of chromium^{51} per ml of suspension. At the end of a 90-min. incubation period ascorbic acid (2 mg per ml) was added. The cells were then separated from the saline by centrifugation, washed 3 times with fresh isotonic saline and resuspended in the donor's plasma-ACD mixture adjusting the hemoglobin concentration to 12 g/100 ml.

For both the hemoglobin solutions and erythrocyte suspensions the subsequent procedures were as follows: A 100% specimen was obtained by diluting 1 ml of the original material to 100 ml in water, from which a 4 ml counting specimen was removed and placed in a screw-top vial. Individual 1 ml aliquots of the original material were placed into Visking dialysis tubing. The specimens were dialyzed against isotonic saline for periods of 16 to 118 hours. The dialysis fluid was changed 3 times per 24 hours. One dialysis bag was removed at 16 hours, and one at each 24 hours thereafter. The contents of the dialysis bags were transferred quantitatively by multiple water rinses to final 1:100 dilutions. Four ml specimens of the final solutions were used in counting. Six duplication experiments showed the possible error of the method to be 3%. The 4 ml specimens were counted in a well-type scintillation

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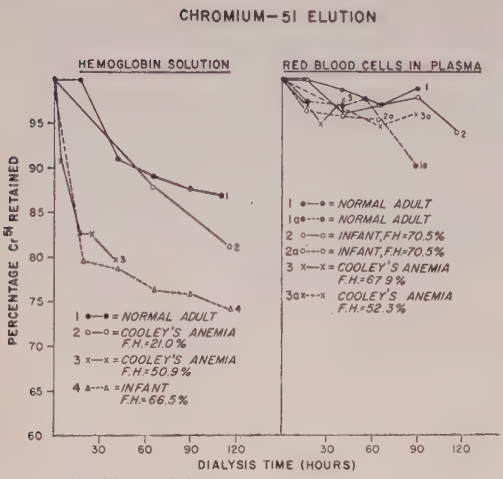

F.H. = fetal hemoglobin

FIG. 1. Rates of chromium[51] elution from hemoglobin solutions and red blood cells from normal adults, infants, and patients with Cooley's anemia when dialyzed against isotonic NaCl.

counter and correction for radioactive decay was accomplished by recounting the 100% specimen concomitantly with each subsequent specimen.

Results. Hemoglobin solutions were obtained from one adult (fetal hemoglobin = <2%), one infant (fetal hemoglobin = 66.5%), and 2 patients with Cooley's anemia (fetal hemoglobin = 21% and 50.9%). As may be seen in Fig. 1 (left), a markedly greater rate of elution of chromium[51] occurred from the infant's than from the adult's specimen. Elution from the specimen containing 21% fetal hemoglobin obtained from a patient with Cooley's anemia was only slightly greater than that from the adult's specimen; that from the patient with Cooley's anemia containing 50.9% fetal hemoglobin was almost as rapid as that occurring from the infant's specimen. Therefore rapid rates of elution occurred in specimens containing fetal hemoglobin whether obtained from an infant or from patients with Cooley's anemia. The rate of elution appeared to be directly related to the percent fetal hemoglobin present.

Intact tagged erythrocytes were prepared from blood specimens from 2 normal adults (fetal hemoglobin = <2%), from 2 infants (fetal hemoglobin = 70.5 and 70.5%), and from 2 patients with Cooley's anemia (fetal hemoglobin = 52.3% and 67.9%). The rapid rates of elution found with hemoglobin solutions were not demonstrated; the content of fetal hemoglobin within the erythrocyte produced no significant difference in rate of chromium elution. (Fig. 1, right).

Discussion. Chromium[51] tagged red blood cells have been used extensively for estimation of erythrocyte longevity in many disorders including the abnormal hemoglobin syndromes and Cooley's anemia(3-4). Elution of chromium[51] from tagged normal erythrocytes has been investigated *in vivo* by simultaneous erythrocyte survival studies comparing the chromium[51] technic and the Ashby method of differential agglutination, and *in vitro,* by dialysis(5-9). However, elution has not been studied in suspensions of cells containing abnormal hemoglobins or fetal hemoglobin.

Hollingsworth reported that estimates of mean life span of fetal erythrocytes were shorter using the chromium[51] technic than those previously reported utilizing the method of differential agglutination(1). Since the life span of fetal erythrocytes could not be correlated with the percent fetal hemoglobin present in the cells, it is unlikely that the differences were secondary to aberrations in chromium elution from the cells resulting from the presence of fetal hemoglobin. Data available for comparison of erythrocyte longevity by the chromium[51] and differential agglutination methods in patients with Cooley's anemia have not demonstrated differences in results obtained by the 2 technics. The 50% survival times of erythrocytes from 3 untransfused patients with homozygous intermediate Cooley's anemia were reported by Kaplan and Zuelzer as 21, 22, and 27 days by the Ashby method(10). The 50% survival times in 3 comparable patients by the chromium[51] technic were reported from this laboratory as 19, 22, and 27 days(4). Corrections for elution were based on rate of elution previously demonstrated for normal erythrocytes.

Suderman *et al.* observed by *in vitro* dialysis experiments that chromium did elute more rapidly from solutions of hemoglobin ob-

tained from cord specimens than from specimens from normal adults(2). This has been confirmed in the present studies. Elution rate has been shown to be directly related to percent fetal hemoglobin present. Rapid rates of elution were also present in hemoglobin solutions containing fetal hemoglobin obtained from patients with Cooley's anemia. Elution from intact erythrocytes has previously not been investigated. In the present studies no clear cut difference in elution could be demonstrated between labeled intact red blood cells from adults, infants, and patients with Cooley's anemia.

Elution of chromium from normal erythrocytes has been shown to occur at a level of approximately 1% per day(5-9). It would be difficult to demonstrate a significant difference in rate of elution from erythrocytes of normal adults and from cells containing large percentages of fetal hemoglobin by dialysis where the error of the method may be of the same order of magnitude as the possible difference in rate of elution. Evidence thus far does not support the existence of any influence by fetal hemoglobin on rate of chromium elution from intact erythrocytes. A final solution to this problem might be obtained by studies of survival of erythrocytes from patients with Cooley's anemia in normal recipients simultaneously by the differential agglutination and chromium^{51} technics.

Summary. Accelerated rates of chromium^{51} elution from hemoglobin solutions containing fetal hemoglobin have been shown to be present in specimens obtained from an infant and from patients with Cooley's anemia. This acceleration has been shown to be directly related to the percent fetal hemoglobin present. Rapid rates of chromium^{51} elution could not be demonstrated to occur from intact labeled erythrocytes containing large amounts of fetal hemoglobin studied by the technic of dialysis.

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Inhibition of Bilirubin Conjugation *in vitro.** (24285)

WILLIAM J. WATERS, ROBERT DUNHAM, AND WILLET R. BOWEN
(Introduced by L. K. Diamond)
Department of Pediatrics, State University of New York College of Medicine at Syracuse

In recent years we have been interested in the problem of bilirubin metabolism in the newborn and the mechanism of production of bilirubin encephalopathy as well as its prevention(1). Recent studies have suggested an impairment of substrate production and conjugation of bilirubin as a glucuronide in the newborn period(2). In the light of this knowledge, the reports of jaundice associated primarily with 30 mg or more of Synkavite in 2-3 kg newborn infants(3,4) stimulated us to assess the possible role of the latter in inhibition of the conjugation mechanism leading to hyperbilirubinemia. Recent reports would also suggest another possible mechanism of action, namely, increased hemolysis of newborn red cells(5). The report of Silverman *et al.*(6), further stimulated us to test a num-

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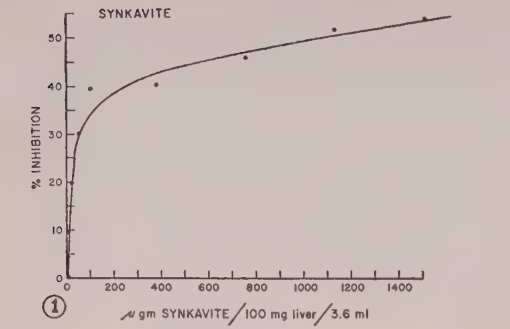

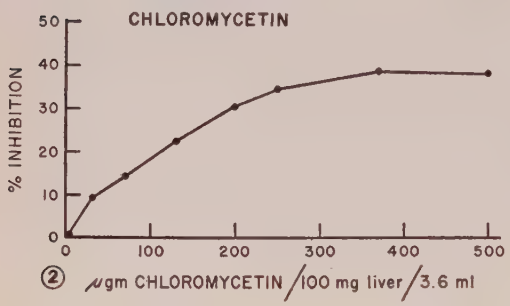

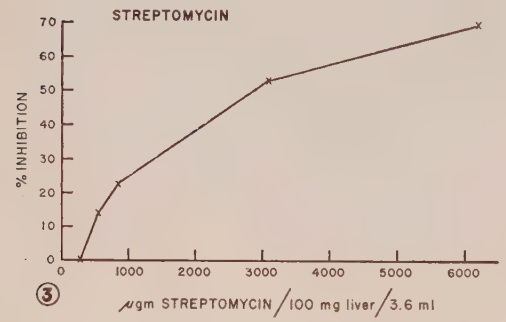

GRAPH 1. % inhibition of bilirubin conjugation in presence of increasing concentrations of synkavite.

GRAPH 2. % inhibition of bilirubin conjugation in presence of increasing concentrations of chloromycetin.

GRAPH 3. % inhibition of bilirubin conjugation in presence of increasing concentrations of streptomycin.

ber of antibiotics and chemotherapeutic agents regarding the possible role of these compounds in the conjugation mechanism.

In vitro studies were carried out using procedures identical to those previously published by others. The preparation and treatment of the liver extracts and homogenates were as outlined by Dutton and Storey(7). The diazoatation was a modification by Lathe (personal communication) of the van den Bergh reaction.

Our objective was to determine the amount of conjugated bilirubin produced in a given incubation and possible inhibition of conjugation as a result of the presence of these potential inhibitors. The amount of conjugated bilirubin in 2 runs was determined, one with and one without the competing substrate. Otherwise, standard conditions were maintained in the 2 flasks and the variation in amount of conjugated bilirubin produced on incubation was assumed to be due to the potential inhibitors.

In all compounds investigated at least 2 series were run to determine the inhibition curves. Reproducibility of inhibition in any series using the same inhibitor and different rats' liver as a substrate and enzyme source was not possible. However, usually the variation did not exceed 10% and this was assumed to be due to varying concentrations of substrate and enzyme in the individual rat liver.

In the newborn, one might expect serum concentrations of at least 20-40 μg/ml of chloromycetin and streptomycin in the usual dosage range (Wehrle, personal communication).

Results. The effect of Synkavite is shown in Graph I. There is an immediate sharp rise in inhibition with a slight increase beyond 250 μg. Initial inhibition is detected at 5 μg and the curve flattens out at 1500 μg.

The effect of chloromycetin is shown in Graph II. The curve represents an average obtained in 2 runs. Initial inhibition is detected at 30 μg with some increase up to 250 μg with a subsequent stabilization.

The effect of streptomycin is shown in Graph III. The curve represents an average obtained in 2 runs. Inhibition is first detected at 560 μg with a fairly steep rise at very high concentrations.

Penicillin, tetracycline, erythromycin, gantrisin, and hydrocortisone show essentially no inhibition in the concentrations investigated which are listed in Chart #1.

Conclusion. Since serum levels are unknown in relation to Synkavite, the data are difficult to interpret but it would appear that inhibition *in vitro* occurs at concentrations which

CHART I. Non-Inhibitors.

Compound	Conc./flask
Penicillin G	0–400 μ
Tetracycline	0–400 μg
Erythromycin	0– 1 mg
Gantrisin	0– 16 mg
Hydrocortisone	0–500 μg

might be expected in an infant, particularly when high doses are given. Chloromycetin shows slight inhibition within the accepted range and streptomycin just beyond the accepted range of serum levels.

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Inhibition of Diaminopimelic Acid Decarboxylase Activity in *Mycobacterium tuberculosis* by Isonicotinic Acid Hydrazide.* (24286)

Hilda Pope Willett (Introduced by D. T. Smith)
Department of Bacteriology, Duke University Medical Center, Durham, N. C.

In an attempt to determine the mechanism of action of isonicotinic acid hydrazide (INH) on *Mycobacterium tuberculosis* attention has been directed in many studies toward an interference with the operation of essential enzymes. Inhibition of growth by INH can be neutralized competitively by pyridoxal, which the drug structurally resembles(1,2). A number of enzyme systems requiring pyridoxal phosphate as coenzyme have also been shown to react with INH(3-5). Although these studies suggest an antagonism, there is as yet no direct evidence that INH exerts its antituberculous activity by inhibiting an enzyme activated by pyridoxal phosphate.

In a recent study in our laboratory it was found that growth of tubercle bacilli in the presence of INH leads to a marked decrease in amount of lysine produced, and that lysine synthesis by the INH-resistant strain is also lower than that by the INH sensitive organism. Since one of the major pathways of lysine synthesis in microorganisms is by way of the pyridoxal phosphate-requiring enzyme, diaminopimelic acid (DAP) decarboxylase, we have investigated the possibility that this enzyme is operative in tubercle bacilli and that it is inhibited in the presence of INH.

Methods. The RlRv strain of *Myco. tuberculosis*, kindly furnished by Dr. William Steenken, was used as the source of enzyme in all of our studies. Cells were harvested after a 3-week growth period on the surface of Proskauer-Beck synthetic liquid medium. They were washed twice in a refrigerated centrifuge with sterile distilled water, resuspended in a small amount of 0.1 M phosphate buffer and ground in a Ten Broeck tissue grinder. Additional buffer was added, the cells recentrifuged, and suspended in a known quantity of buffer. The cells were disrupted in a Raytheon 9KC sonic oscillator followed by centrifugation at 16,000 x G for 30 min. The straw-colored supernatant contained the active enzyme and was used immediately. All operations were carried out in the cold and because of the long reaction time employed, sterile conditions were maintained throughout all the experiments.

The reaction mixture for detecting DAP decarboxylase activity contained 12.5 μM synthetic DAP, 10 μg pyridoxal phosphate,

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TABLE I. Inhibition of DAP Decarboxylase by INH.

INH conc. (μg)	A μg lysine	A % inhibition	B μg lysine	B % inhibition
100	4.3	98.3	34.4	86.4
10	121.7	51.8	175.2	30.7
1	187.9	25.7	227.2	10.2
.1	196.8	22.2	234.4	7.5
Control	252.9			

A = 1 hr preincubation with INH before addition of substrate.
B = No preincubation with INH.

1.0 ml of cell-free extract in a 0.1 M phosphate buffer, pH 7.0. Total reaction volume was 2.0 ml. Suitable controls were included in all experiments. Varying concentrations of INH were employed and in some vessels INH was preincubated with the enzyme preparation before the substrate was added. The reaction time was 18 hours, and the reaction stopped by heating at 100°C for 5 min. The precipitated protein was removed by centrifugation and the clear supernatant solutions used for detection of enzymic reaction products. Presence of lysine was detected by one-dimensional paper chromatography using phenol-water and butanol-acetic as solvents. Final identification of lysine was by use of the microbiological assay method using *Leuconostoc mesenteroides* as the test organism.

Results. Examination of the reaction mixture showed that DAP was converted to lysine by an enzyme present in cell-free extracts of tubercle bacilli. Identification of lysine as the end-product was made by comparison of the unknown ninhydrin-reacting spot with a lysine control in different solvents, and by demonstration of its utilization by the lysine-requiring bacterium, *L. mesenteroides*. No lysine was produced in the absence of DAP. No spot comparable to that of cadaverine was detected by chromatography.

INH was found to cause a marked reduction in activity of DAP decarboxylase. This inhibition was most pronounced when the drug was preincubated with the enzyme-containing extract before addition of substrate. Table I shows the effect of preincubation of the enzyme for 1 hour with INH before addition of the substrate, in a system in which extraneous pyridoxal phosphate was omitted.

When pyridoxal phosphate is added to the reaction mixture, degree of inhibition at the various drug levels is reduced. This protection against INH is greatest when the enzyme is preincubated with pyridoxal phosphate for 1 hour before addition of the substrate and INH (Table II). It is also more pronounced at the lower levels of INH. At the higher concentrations of the drug, the amount of pyridoxal phosphate employed was probably insufficient to neutralize effectively the large dose.

Discussion. Cell-free extracts of *Myco. tuberculosis* strain RlRv have been found to contain an enzyme which decarboxylates α-ε-diaminopimelic acid to CO_2 and lysine. This amino acid, first described by Work in acid hydrolysates of *Corynebacterium diphtheriae* (6,7), has since been shown to be present in many bacteria and blue-green algae, but to be absent from other organisms so far examined (8). Evidence is also accumulating to suggest that DAP serves a metabolic role as a precursor in the synthesis of lysine (9). It has been described and isolated from *Myco. tuberculosis* (10,11) where it is an important fraction of the cell wall, but there has been no previous report of a metabolic role in this organism. Rate of decarboxylation of

TABLE II. Effect of Pyridoxal Phosphate on INH Inhibition of DAP Decarboxylase Activity.

INH conc. (μg)	A μg lysine	A % inhibition	B μg lysine	B % inhibition	C μg lysine	C % inhibition
100	13.2	96.6	70.9	82.2	63.9	83.6
10	267.3	31.5	370.4	7.1	316.9	18.9
1	363.5	6.8	382.1	4.2	364.0	6.7
.1	373.6	4.3	396.5	.58	370.3	5.1
0	390.3		398.8		390.3	

A = 1 hr preincubation with INH before addition of substrate and pyridoxal phosphate.
B = 1 hr preincubation with pyridoxal phosphate before addition of substrate and INH.
C = Substrate, pyridoxal phosphate, and INH added simultaneously.

DAP by cell-free extracts of *Myco. tuberculosis* was increased by addition of pyridoxal phosphate, although it was not essential for enzyme activity. This is probably because with the methods employed, the decarboxylase present in the extract is still largely undissociated from its coenzyme. DAP decarboxylase activity was markedly inhibited by INH. Inhibition was greater if the drug was preincubated with the cell-free extract before addition of substrate. The results obtained also clearly indicate a relationship between toxicity of INH and pyridoxal phosphate. Further studies, however, are necessary to determine whether the reaction is a strictly competitive one between INH and pyridoxal phosphate for active sites on the enzyme molecule. The protection obtained by preincubation of the preparations with pyridoxal phosphate prior to addition of INH seem to indicate a competitive relationship.

On the basis of our findings it is suggested that the DAP decarboxylase is important in the metabolism of *Myco. tuberculosis*, serving as an important and probably major pathway in lysine synthesis. The finding that DAP decarboxylase is inhibited by INH, and that this inhibition can be overcome by pyridoxal phosphate, is in accordance with the previous observation in our laboratory that lysine synthesis by *Myco. tuberculosis* is markedly inhibited by culture in the presence of subinhibitory concentrations of isoniazid. Because of the nature of the isoniazid molecule the interpretation of any data relating to its specific mode of action is difficult, but the finding of a pyridoxal phosphate-requiring enzyme whose activity is inhibited by the drug lends additional support to the hypothesis that one of the important points of attack of INH in *Myco. tuberculosis* are those enzyme systems requiring pyridoxal phosphate as coenzyme.

Summary. DAP decarboxylase activity has been detected in cell-free extracts of *Myco. tuberculosis*. Enzyme activity was inhibited by INH and this inhibition could be overcome by pyridoxal phosphate.

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Effect of d-Lysergic Acid Diethylamide on Spinal Reflexes. (24287)

LESLIE E. GEIGER AND PETER CERVONI (Introduced by William H. Chambers)
Clinical Research Division, U. S. Army Chemical Warfare Laboratories, Army Chemical Center, Md.

The alterations in mental function induced by d-lysergic acid diethylamide (LSD) are, presumably, paralleled by modifications of the activity of cells or synapses within central nervous system, whether cortical, subcortical or spinal. LSD, when administered to spinal cats, in doses as small as 10 μg/kg i.v. was found to augment polysynaptic withdrawal and crossed extensor reflexes(1). At higher doses (40-80 μg/kg) depression of the flexion reflex was found. The monosynaptic knee jerk was less consistently affected. After administration of LSD in doses sufficient to cause mental symptoms increased activity of stretch reflexes has, however, been a consistent clinical observation(2). In the following experiments, the effects of LSD were evaluated by measurement of changes in magnitude of the reflex volley in ventral roots rather than by recording of muscular contraction.

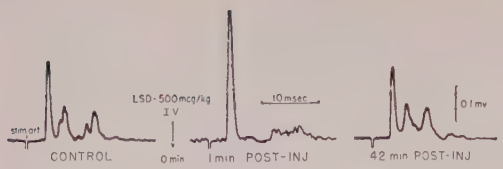

FIG. 1. Oscilloscopic tracing of response in 6th lumbar ventral root in response to stimulation of peroneal nerve.

Methods. The upper cervical spinal cords of adult cats were sectioned through the atlanto-occipital membrane under ether anesthesia. Peripheral nerves and ventral roots were prepared for stimulation and recording utilizing standard technics(3,4). In these experiments a gastrocnemius, tibial or peroneal nerve was stimulated with single square wave stimuli 0.08-3.0 volts intensity and 0.1-0.4 msec. duration. The stimuli were repeated at 2 or 3 second intervals. Temperatures of the animal and the pool of oil surrounding the cord were maintained at 36° and 34°C, respectively. Only those preparations whose baseline responses over a 30 minute control period varied less than 10% are considered in this report (20 out of 37). The criterion of significant change in amplitude of the monosynaptic spike was 20% and the change in area of the polysynaptic complexes, estimated visually, was 50%. Blood pressure was recorded from a common carotid artery with a strain gauge recorder. Thirty-six injections of LSD-25 (Sandoz) dissolved in sterile physiological saline, were administered through a cephalic vein in 20 preparations. Dosage ranged from 4-1000 μg/kg. The usual injection time was 10 seconds. Rate of injection did not influence the results.

Results. The characteristic modification of reflex activity observed in this study is shown in the figure. Increased height of the monosynaptic response was observed after 11 of 16 injections of doses greater than 200 μg/kg. Below this dose only 1 of 20 injections resulted in facilitation. Four instances of failure to respond to doses of LSD greater than 200 μg/kg occurred in cats which had received another dose of LSD less than 2 hours previously. Only one such failure was observed among cats receiving more than 200 μg/kg as the first dose. Increased amplitude of the monosynaptic spike became apparent within 15 seconds after completion of injection and lasted from 4 minutes to 3 hours. Maximum facilitation was observed with 250-300 μg/kg. Higher doses tended only to prolong this effect, but no consistent relation of duration of effect to dose level was observed. For these reasons quantitative analysis of the dose-response relations were not possible. Polysynaptic activity was observed in several preparations where nerves to flexor muscles were stimulated. With one exception, depression of polysynaptic activity accompanied facilitation of the monosynaptic reflex. After injection of doses higher than 100 μg/kg a significant increase in blood pressure was found but facilitation of the monosynaptic spike did not necessarily parallel the hypertensive effects.

Discussion. The foregoing experiments and those previously published on evaluation of LSD action on spinal monosynaptic and polysynaptic pathways(1) are not directly comparable. In the previous studies, muscle reflexes were recorded. In addition to an effect on the central pathway, reflex activity may have been modified by the action of LSD on peripheral structures. Of significance in this respect is the direct peripheral vasoconstriction which LSD induces similarly to its analogue, ergonovine(5). Resulting interference with muscle perfusion may influence contractility and modify the activity of proprioceptive reflexes.

The observation that larger doses were required to produce effects in this series than those reported in previous studies(1) is consistent with the observation of Elder *et al.* (6) that reproducible LSD intoxication in cats is obtained only with intravenous injection of 400 μg/kg or greater.

The magnitudes of the reflexes reported here are dependent primarily upon the integrity and the excitability of motoneurones and, in turn, upon the facilitatory and inhibitory tone of the internuncial systems. Since LSD may act upon any of these components, more precise localization of site of action of LSD is required before interpretation of a central mechanism of action is feasible. At present

significant comparisons can be made, however, with the action of LSD upon other central pathways. Purpura(7) has postulated that those cortical tracts which have predominantly axosomatic synaptic connections are facilitated by LSD. Since the connections of muscle afferents with spinal motoneurones are principally axosomatic(8) facilitation of the monosynaptic spike is to be expected.

Also pertinent is the concept that certain cortical association tracts(9), midbrain reticular pathways(10) and spinal reflexes(11) contain adrenergic links. The present study shows that LSD modifies spinal reflexes in a manner analogous to that of epinephrine(12). Similar evidence has been presented in experiments on the transcallosal preparation(9). LSD may, therefore, interact with proposed adrenaline-like transmitters.

Summary. The amplitude of the monosynaptic reflexes recorded from the ventral roots of unanaesthetized spinal cats was found to be increased after i.v. injection of LSD in doses greater than 200 μg/kg. Polysynaptic activity was depressed coincidentally with facilitation of the monosynaptic spike.

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Zone Electrophoresis of Cerebrospinal Fluid Proteins in Starch Gel.* (24288)

JAMES H. PERT AND HENN KUTT (Introduced by Joseph Reilly)
Second (Cornell) Neurological Service, Bellevue Hospital and Dept. of Medicine, Cornell University Medical College, N. Y. C.

The observation that total protein in cerebrospinal fluid may increase substantially in many diseases of the nervous system has stimulated considerable investigation of the protein fractions with new methods of protein separation. It has been hoped that when specific changes were found, they would be of diagnostic value and would shed some light on the large number of neurologic diseases of unknown cause. The exceptional resolution of serum protein fractions by electrophoresis in starch gel described by Smithies (1), and our(2) experience with this method, whereby we can obtain 15 to 20 protein fractions in normal sera, suggested that comparable fractionation might be gained by applying this technic to cerebrospinal fluid protein.

Methods and materials. Specimens of spinal fluid and serum were obtained from patients in the hospital. Most of these patients suffered from disease of the nervous system; only a few could be considered normal subjects. After determining concentration of protein of spinal fluid by the sulpho-

* This investigation was supported in part by grant from Nat. Multiple Sclerosis Soc. and Special Clinical Traineeship from Nat. Inst. of Neurological Diseases and Blindness.

salicylic acid turbidity method(3), the specimen was concentrated to give an approximate final concentration of 1 g% protein. Concurrently a serum specimen was diluted with 0.9% sodium chloride to 50 or 100 times its original volume and concentrated by the same method as the spinal fluid. The principal method of concentration was dialysis of the specimen in cellophane bags against 35% solution of Polyvinylpyrrolidone (PVP).† Usually 10 ml of cerebrospinal fluid were concentrated to 0.2 ml. To assure recovery of the necessary volume of concentrate, a glass container of appropriate size was incorporated into the bottom of the bag. Containers of 0.2 ml to 2 ml were designed to allow concentration from 1-50 to 1-5. The appropriate amount of granular starch was placed in the container so that after concentration, the specimen was ready for application in the starch gel electrophoresis run. Care was taken to remove any air bubbles from the glass container prior to tying the top of bag. A small weight was attached to bottom of container to keep the bag in the vertical position. This method allows predetermination of volume of final concentrate and minimizes the danger of losing the specimen.

When specimens contain less than 25 mg% of protein, it is necessary to concentrate the material as much as 100 times, and under these circumstances we found lyophilization more satisfactory because of the small volume of final concentrate. Prior dialysis is then necessary to eliminate excess electrolyte so that final electrolyte concentration is approximately the same as that existing before concentration. Lyophilization, however, has the disadvantage of altering lipoproteins and is not necessary with most pathologic specimens, because the protein concentration is usually elevated.

Electrophoresis conditions. Preparation of soluble starch and starch gel was similar to that described by Smithies(1). Details of method of preparing soluble starch for quantitative studies of electrophoresis of serum proteins in starch gel are being published(2). For spinal fluid studies we used a starch which is not hydrolyzed‡ as long as the starch used for serum studies. These investigations utilized Idaho starch, Batch #584, manufactured by Magic Valley Processing Co., Twin Falls, Idaho.§ The gel was prepared by heating 13.5 g of soluble starch with 100 cc of 0.030 M borate buffer pH 9.05. Because of the small amount of protein in the sample, we employed a plastic form with cross-sectional area approximately half that used for routine serum work. It measured 7 mm by 225 mm and was 6 mm deep. Optimal running time was 6 hours at 2 milliamperes/gel strip at 14°C. After completion of electrophoresis, the gel strip was removed from the plastic mold and cut in half longitudinally. One-half was stained with naphthalene black B 200 to visualize the proteins, the other half was stained with Oil Red O to show lipoproteins. The lipoprotein stain was prepared by making a saturated solution of Oil Red O in methanol : acetic acid : water, 60 : 10 : 30. The gel strips were left in this solution for 48 hours with 2 changes of fresh stain. They were then washed with continuous flow of tap water until background staining was reduced to a minimum. Permanent records were made through photography and with a photoelectric reflectometer designed for this purpose.‖ With this instrument a continuous scan of each strip is carried out and a graph of the stained proteins is obtained (Fig. 1,2).

Results. The serum protein patterns were similar to those previously reported(1), and for convenience we have used the same nomenclature when possible. The orientation of fractions in cerebrospinal fluids was similar to that in serum of the same patient (Fig. 1); however, differences were also obvious. The faster of the 2 pre-albumins was always considerably increased over that found in

† Generously supplied by Abbott Laboratories, Chicago, Ill.

‡ Soluble starch for serum studies is prepared by mixing 300 g of preheated starch and 600 cc acetone HCl (100:1) and let stand for 70 min. at 36.4°C. For cerebrospinal fluid studies contact time is reduced 10%.

§ Obtained from Morningstar-Nicol, Inc., N.Y.C.

‖ Generously supplied by Biophysics Dept. of Airborne Instrument Co., Mineola, L.I., N. Y.

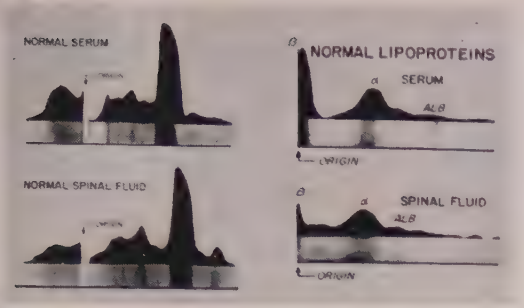

FIG. 1. An example of normal spinal fluid and serum electrophoresis patterns. Strips on left, stained with naphthalene black B, illustrate differences in protein distribution between the 2 fluids. Strips on right were stained with Oil Red O for lipoproteins; their reflectometer curves were made at higher magnification.

serum, as were the bands in the beta area. Concentration of gamma globulin, and to a lesser extent haptoglobins, was less in the spinal fluid. The most marked reduction was in slow alpha-2, the major high molecular weight fraction of alpha-2 globulins. Approximately 25% of spinal fluid samples examined showed a narrow, sharp band which migrated toward the cathode but was clearly separated from the gamma fraction (Fig. 2). This "fast gamma" fraction has not been seen in corresponding sera, has not been reported to exist in sera by other workers who have employed the same technic of electrophoresis, and has not been found in normal spinal fluid examined by us. It has been noted in such diseases as multiple sclerosis, primary lateral sclerosis, thrombosis of posterior inferior cerebellar artery, and seizure disorder of unknown origin, and was noted in one patient in 2 successive samples drawn 2 months apart. A number of abnormal spinal fluid protein patterns are illustrated in Fig. 2. It is not intended to suggest that these protein patterns are characteristic of the disease, but rather to demonstrate types of abnormalities which may be encountered. The pattern in cerebrospinal fluid specimen from a patient with bacterial meningitis shows poor separation of proteins in the alpha-beta region. In addition, 4 pre-albumin fractions are noted. Since only 2 pre-albumin fractions have been noted in any other condition, this latter finding may be of significance. The multiple sclerosis specimen illustrates changes in gamma as well as increases in post-albumin globulins. In the specimen from patient with Guillain-Barre Syndrome, a striking increase in gamma globulin is noted.

Lipoproteins appeared in 2 areas on the gel strip (Fig. 1). One, close to the origin, consisted of a wide zone with dense front migrating toward the anode. The other migrated in the post-albumin region. By experiments with isolated lipoproteins, the fraction close to the origin was identified as beta lipoprotein (low density lipoprotein floating in medium of density of 1.063); the fraction close to the albumin corresponded to alpha lipoprotein (high density lipoprotein which sediments in bottom of tube in medium of 1.063 density). Changes in alpha and beta lipoprotein fractions in spinal fluid generally followed alterations noted in the serum. The only consistent observation, however, is that the beta lipoprotein in spinal fluid is relatively decreased compared to that found in serum. The lipoprotein patterns of a normal serum and cerebrospinal fluid are illustrated in Fig. 1.

Discussion. When free electrophoresis(4, 5,6,7) and paper electrophoresis(8,9,10) were employed in separation of cerebrospinal fluid protein, albumin, alpha-1, alpha-2-, beta-, and gamma globulins were regularly noted. When concentration is adequate, 2 fractions, X and X-1, may be seen to migrate in front of albumin(5,6). In addition, some investigators

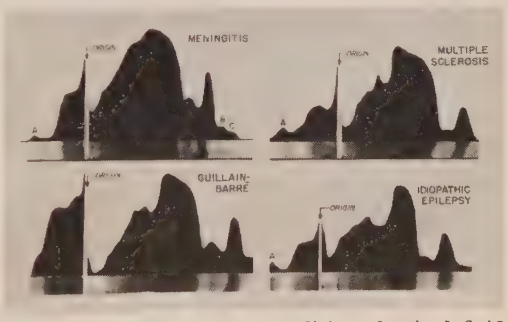

FIG. 2. Examples of abnormalities of spinal fluid protein distribution in 4 disease states. The "fast gamma" (see *A* above) can be seen in specimen of bacterial meningitis, multiple sclerosis, and idiopathic epilepsy migrating to left and separate from gamma. The meningitis shows 2 additional pre-albumins (see *B* and *C* above).

have noted other fractions which migrate in the beta-gamma region, and have designated them as *tau*(6,8,11), *phi*(7,12), and gamma-2 (13). Success in demonstrating the X, X-1, tau, phi, and gamma-2 fractions has been variable, depending upon method employed. There is general agreement, however, that there are few, if any, characteristic changes in protein pattern in cerebrospinal fluid in disease states.

When specific changes are demonstrated, we might expect that they will be revealed by a technic which separates the proteins into individual fractions rather than into groups. Although neither our technic nor any other technic reported provides for such precise fractionation, the method herein described may be a step in this direction.

In spinal fluid electrophoresis it is difficult to utilize the full potential of this method of protein separation because the total sample may be as little as 1 mg. If electrophoresis were carried out in the manner which would produce optimal fraction separation in serum, many low concentration fractions in spinal fluid would not be demonstrable. A compromise between fraction identification and fraction separation must be made. To accomplish this we have used a somewhat underhydrolyzed starch, a 6-hour rather than a 16-hour running time, and a plastic form with a cross-sectional area somewhat less than half of that used for routine serum work. As a result of changing the technic, we can regularly identify only 10 to 12 fractions in serum or spinal fluid rather than 15 to 20 obtainable with the larger protein application available with serum under optimal electrophoresis conditions. We do not know how critical this limitation will be in detecting specificity of protein change in disease states.

An additional problem in determining specificity of protein distribution in disease states, is the desirability of quantitation. With paper electrophoresis we have shown that as total protein increases, the percent of each fraction increases at different rates (unpublished). Without quantitation, this factor cannot be taken into account and only large protein alterations of fractions normally present will be distinguishable from normal variation. As used at present, however, the starch gel method is useful for exploring occurrence of qualitative changes in protein in disease states.

Changes of lipoprotein patterns in cerebrospinal fluid have been noted by other investigators(10,14,15). In starch gel electrophoresis, lipoproteins migrate in areas where no major protein components are present (Fig. 1). This is an advantage since it aids in differentiating lipoproteins from high concentration protein fractions which may have some yellow color unstained and may adsorb small amounts of Oil Red O.

With the method described, no fractions have been observed in normal spinal fluid which were not present in the serum of the same patient. However, in abnormal spinal fluid, fractions have been noted which were not detected in the sera. Starch gel electrophoresis does confirm the observation from other methods that there are significant differences in relative concentration of the fractions in the 2 fluids. The low concentration of slow alpha-2 suggests that large molecular size is a factor in determining which protein can cross the blood-brain barrier. This relationship, however, is a complex one.

Summary. 1. A method for separation of cerebrospinal fluid proteins by electrophoresis in starch gel is presented, and its advantages and limitations are discussed. 2. In normal spinal fluid 10 to 12 protein fractions are observed. These correspond to serum proteins in the same individual but are present in different relative concentrations. In a number of cerebrospinal fluids from patients with neurologic diseases, considerable alterations of protein patterns were noted. Some examples of the type of abnormalities which may be encountered are illustrated. 3. In some of these pathologic specimens protein fractions were found, which we have not yet seen in serum or in normal spinal fluid. These have been designated as "fast gamma" and third and fourth prealbumins. 4. A method for concurrently staining lipoproteins in cerebrospinal fluid and serum has been described.

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Sulfhydryl Content of Failed and Strophanthin Poisoned Rabbit Hearts. (24289)

ROBERT C. REYNOLDS, MAYNARD B. CHENOWETH AND GEORGE L. ELLMAN
Biochemical Research Lab., The Dow Chemical Co., Midland, Mich.

Recently, Bertelli and Mussini(1) reported that the sulfhydryl content of hearts perfused with 1:100,000 strophanthin-g (ouabain) was markedly lowered. There was a decrease of approximately 50% of sulfhydryl content of hearts in complete systolic contracture induced by strophanthin-g, compared to unpoisoned, perfused rabbit hearts. This finding and observations in our laboratory, led us to study the sulfhydryl content of crude myosin extracts of hearts after the hearts had been subjected to various conditions.

Methods. Perfusion. Rabbits of the same strain, 3 to 4 months old, raised in air conditioned animal room on the same diet, were killed by blow on occiput, and their hearts removed as rapidly as possible. These hearts were perfused with the apparatus described by Anderson and Craver(2), available from Metro Industries, L. I. City, N. Y. The perfusion solution was described by Chenoweth and Koelle(3). This work was performed during the summer months. Immediately after removal, the hearts were perfused 30 minutes at 37 $\pm$ 0.5°C. The first 500 ml of perfusion solution passing through heart was not recirculated because it contained blood. To avoid possible injury to hearts by attaching a hook, kymograph recordings were not made. Hearts that appeared damaged or showed evidence of failure during this 30-minute period were not analyzed. After perfusion, the drug was added to a liter of solution in the aeration reservoir, and the heart perfused 15 minutes more. Each heart was perfused 45 minutes. A control group of hearts was perfused 45 minutes continuously. A second group was perfused with 1:100,000 strophanthin Kombé N. F. (S. B. Penick Co.) for 15 minutes. Then they were in complete systolic contracture. A third group was perfused with 0.04% sodium pentobarbital. A fourth group was perfused with 5% ethanol (v/v). A fifth group was failed by perfusing with a solution containing 25% of usual calcium ion concentration: 0.54 mM $CaCl_2$/l, or 0.079 g $CaCl_2 \cdot 2H_2O$/l. All hearts in the last 3 groups failed, stopping in diastole in 7 to 9 minutes; however, all hearts were perfused for the full 15 minute period. *Extraction.* The hearts were removed from apparatus and ventricles were immediately frozen in liquid nitrogen. While still frozen, the ventricles were crushed in stainless steel

TABLE I. Rabbit Heart Sulfhydryl Content (μg SH/mg N).

	Strophanthin-K poisoned	Untreated	Failed Pentobarbital	Failed Ca^{++} deficient
			13.95	
	17.	15.52	13.01	17.27
	18.05	16.	16.05	14.20
	18.06	16.58	14.15	14.04
	18.75	17.68	11.35	17.62
	18.52	16.26	11.85	17.61
Mean	18.08	16.41	13.39	16.15
S.D.	.06	.72	1.72	1.66
P	.008		.006	

cylinder by sledge-hammer driven piston. Then portions weighing 100 to 200 mg were suspended in 10 ml of 3.6% potassium chloride—0.4% potassium bicarbonate solution with a Potter-Elvejhem homogenizer equipped with Teflon® pestle. This procedure dissolves myosin and other water soluble fractions of heart but not fibrous tissue. This crude extract of heart muscle was centrifuged 30 minutes at 6,000 rpm at 5°C. The supernatant was immediately analyzed for sulfhydryl content by the method described by Ellman(4). Micro-Kjeldahl determinations for nitrogen were performed on an aliquot of this supernatant. *Sulfhydryl determinations*: Two ml of supernatant were diluted with 3 ml water and 2 ml 0.03 M potassium phosphate buffer at pH 8.0. To this solution was added 3 ml of 0.1% acetone solution of bis (*p*-nitrophenyl) disulfide. The mixture was then centrifuged 5 minutes at 6,000 rpm to remove precipitated protein. The yellow *p*-nitrobenzenethiol anion which was released by the sulfhydryl groups in the tissue was then measured with Beckman DU spectrophotometer at 412 mμ. These determinations were made against appropriate blank solutions. Amount of sulfhydryl present was determined and sulfhydryl content/mg of nitrogen calculated. The data were analyzed statistically with the "t" test according to Snedecor(5).

Results. Table I presents data. The significant changes are (1) depression of SH levels in pentobarbital failed hearts, and (2) elevation in Strophanthin K poisoned hearts. Failure caused by treatment with Ca deficient bath fluid failed to change SH levels. Measurements on hearts failed with ethanol are not given in the Table since variation in results is so large as to make comparison with other results difficult. [12.73 ± 5.4 (1 SD) mg SH/mg N]. We did not at any time observe detectable SH material in the perfusion fluid.

Discussion. These data are obviously in contradiction to data of Bertelli and Mussini, who reported a lowering of sulfhydryl concentration with strophanthin-g poisoning. and these data indicate a rise in sulfhydryl concentration with strophanthin Kombé poisoning. Since both of these drugs are very similar pharmacologically, it seems strange that they should produce opposite effects on sulfhydryl levels.

Our data also suggest that in some types of failure, sulfhydryl concentration of heart muscle is lowered. Since Bertelli and Mussini did not describe the condition of hearts before adding strophanthin-g, it is possible that the hearts were already in some degree of failure before the drug was added. These workers did not analyze hearts for sulfhydryl that had been failed. The methods of sulfhydryl determination in these experiments, although different, are both reliable methods; therefore, there should be no disagreement arising from actual analysis of sulfhydryl groups. It is more likely that a difference would arise from the methods in which tissues were handled before analysis. Bertelli and Mussini do not describe the procedure they followed in homogenizing their tissue samples, but care has been taken, in this case, to preserve the sulfhydryl content of tissues during analysis.

Summary. Perfused rabbit hearts were analyzed for sulfhydryl content. Unfailed hearts showed average concentration of 16.41 μg sulfhydryl/mg nitrogen. Hearts which had been put into systolic contracture with strophanthin-K showed a significant rise in sulfhydryl concentration, whereas hearts which were put into diastolic arrest with sodium pentobarbital showed a significant lowering in sulfhydryl concentration. Hearts which were put into diastolic arrest by lowering of calcium ion in the perfusion media showed an average sulfhydryl concentration

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Adrenocortical Function in Some Mental Diseases. (24290)

DHIRENDRA NATH NANDI AND SACHCHIDANANDA BANERJEE
Department of Physiology, Presidency College, Calcutta, India

Controversial reports have appeared regarding the functional state of the adrenal cortex in mental diseases. In schizophrenia, hypoactivity of the adrenal cortex has been claimed(1-6) and denied(7-10). One reason for this controversy might have been the different diagnostic criteria of the workers. The present investigation deals with functional activity of the adrenal cortex in patients suffering from schizophrenia, paranoia, depression and mania. Eosinopenic response to injected epinephrine was used as the test in all the diseases. In some of the schizophrenic patients eosinopenic response to injection of ACTH and urinary excretion of 17-ketosteroids were also determined.

Methods. Patients were selected from Lumbini Park Mental Hospital at Calcutta. Normal subjects were male and female nurses of the same hospital. Patients with composite diagnosis like depression with paranoia, patients complicated with arteriosclerosis, psychosis, general paralysis of the insane, thyrotoxicosis with psychosis were avoided. The 4 groups of patients were selected according to the following basic features: *Schizophrenia* starts insidiously at adolescence and progresses slowly. There is gradual personality deterioration with disorders of feeling, thought, conduct and memory, and withdrawal of interest from environment. *Paranoia* is a focal personality disorder with delusion formation without much deterioration of personality. Hallucination may be present. Power of reasoning remains relatively unimpaired, but does not accord with the existing surroundings and patients do not realize the absurdity of their delusions or hallucinations. *Mania* is associated with elated mood with emotional instability. There is flight of ideas and psychomotor hyperactivity. Megalomanic ideas may be present. *Depression.* Patients suffer from depressed mood and retardation of thinking process. Sluggishness of psychomotor activity, sense of guilt, self-reproach and self-depreciating ideas are often present. In the schizophrenic group stress was given to the basic features, and different sub-classes of this group were not included. In manic depressive psychosis, importance was given to the nature of manifestation at time of study and patients of this group were included in either mania or depression. .3 cc of epinephrine hydrochloride (1/1000) was injected subcutaneously in early hours of morning before breakfast and blood collected from anticubital vein in oxalated tubes both before and 4 hours after injection. Eosinophils in blood samples were counted by the method of Menneman *et al.* (11). In 8 schizophrenic patients, who did not improve with insulin shock and electric convulsion therapy, 25 mg ACTH was injected subcutaneously and eosinophils were counted in the blood samples collected before and 4 hours after the injection. In these patients 17-ketosteroids in 24 hour urine samples were extracted and estimated by the method of Davidson *et al.*(12).

Results. Tables I-III summarize the results. Eosinophils of patients suffering from schizophrenia and mania were significantly

TABLE I. Blood Eosinophils and Their Reduction 4 Hours after Subcutaneous Injection of .3 cc Epinephrine Hydrochloride (1/1000).

Subjects	Eosinophils/cm blood before epinephrine	t	Reduction of eosinophils (%) after epinephrine	t
Normal (15)	386 ± 32*		63 ± 2	
Schizophrenia(26)	498 ± 37	3.1	31 ± 3	10
Paranoia (21)	300 ± 22	2.3	63 ± 2	0
Depression (10)	188 ± 32	4.5	75 ± 3	3.3
Mania (15)	566 ± 75	2.3	24 ± 3	11.8

* Mean ± stand. error.

Figures in parentheses indicate No. of subjects.

TABLE II. Blood Eosinophils and Their Reduction 4 Hours after Subcutaneous Injection of 25 mg ACTH.

Subject	Eosinophils/cm blood before ACTH	Reduction of eosinophils (%) after ACTH
Normal (15)	380 ± 31	63 ± 2
Schizophrenia (8)	622 ± 126	31 ± 6

TABLE III. 24-Hour Urinary Excretion of 17-Ketosteroids (mg).

Subjects	24-hr urinary 17-Ks (mg)
Normal (15)	16.94 ± .91
Schizophrenia (8)	3.39 ± .63
t	12.3

higher than the counts in normal persons. Eosinopenic response to injections of epinephrine was significantly lower in patients suffering from schizophrenia and mania, higher in depression, and did not differ from normal subjects in patients suffering from paranoia. Eosinopenic response to injection of ACTH was lower in schizophrenia. Urinary excretions of 17-ketosteroids were significantly lower than those of normal Indians(13).

Discussion. Schizophrenia. All patients had possibly hypofunctioning adrenal cortex as evidenced by eosinopenic response to epinephrine and ACTH and urinary excretion of 17-ketosteroids. Epinephrine may directly depress the eosinophil count as has been observed by Thorn(14) but the depression was less in schizophrenics than normal subjects. ACTH also produced less depression of eosinophil count in schizophrenics than normal persons. Eosinopenic response to epinephrine and ACTH was qualitatively similar in both schizophrenics and normal persons. It is, therefore, likely that epinephrine acted indirectly through the pituitary by liberating ACTH and eosinopenic response to epinephrine may be considered as a suitable test for determination of functional activity of the adrenal cortex in schizophrenia. Average daily urinary excretion of 17-ketosteroids in patients suffering from schizophrenia was 3.39 mg; corresponding excretion in normal subjects of the same economic and social status was 16.94 mg. Patients were between 21 and 35 years of age. The depressed output of 17-ketosteroids was, therefore, possibly not due to the aging process(15). It, however, might have been a non-specific reflection of chronic disease. The contradictory reports on functioning of the adrenal cortex by various workers might be due to the different diagnostic criteria followed in placing a patient suffering from mental diseases under schizophrenia. Patients suffering from mania also showed diminished activity of adrenal cortex. The finding was similar to that reported by Lehman *et al.*(16). Symptoms similar to those of mania are usually seen in prefrontal leucotomised subjects. Vargha *et al.*(17) observed that after leucotomy operation eosinophils of blood increased. It might be that leucotomy depressed functional activity of the adrenal cortex. As the pituitary-adrenal axis is regulated by the hypothalamus, a disorder in the relation between prefrontal cortex and hypothalamus in mania might be responsible for the malady.

The type of mental reaction in depression is the reverse of mania. Hyperactivity of the adrenal cortex was observed in patients suffering from depression. From the psychological point of view superego is hyperactive in cases of depression. The somatic structure responsible for superego activity in all probability lies in the prefrontal area of the cerebral cortex. The symptoms of depression might be due to hyperactivity of prefrontal cortex. In depression, therefore, the possible site of depression might be in the cortical hypothalamic relation leading to increased pituitary

adrenocortical activity. The functions of adrenal cortex were normal in paranoia. In this disease the psychical disturbance is limited within certain spheres of mental organization and condition of stress is less in comparison with other psychotic diseases. The functional activity of the adrenal cortex, therefore, possibly did not change. Diminished urinary excretion of 17-ketosteroids in schizophrenia might be due to hypofunction of testes Leydig cells, of adrenal cortex or of both these organs. Hemphill *et al.*(18) observed degenerative changes in the testes of schizophrenics and changes in most patients were similar to those of the testes of hypophysectomized animals. Urinary excretions of 17-ketosteroids also decreased both after electric convulsion therapy(18,19) and prefrontal leucotomy (18). The disorder in schizophrenia seems likely to be in the cerebral control of pituitary.

Summary. Eosinopenic response to injected epinephrine was studied in patients suffering from schizophrenia, paranoia, depression and mania. Eosinopenic response was lower in cases of schizophrenia and mania, higher in depression and normal in paranoia. Eosinopenic response to injected ACTH which was studied in 8 schizophrenic patients was very low. 24-hour urinary excretion of 17-ketosteroids by these schizophrenic patients was also very low.

We are indebted to Dr. T. C. Sinha, Superintendent of Lumbini Park Mental Hospital of Calcultta, for kind permission to work with patients of the hospital.

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Potentiating Effects of Reserpine on Thyrotoxicity in the Rat.* (24291)

BENJAMIN H. ERSHOFF

Department of Biochemistry and Nutrition, University of S. California, Los Angeles

Considerable data are available indicating that reserpine under various experimental conditions has an anti-thyroid effect. Thus Kuschke and Gruner(1) reported that large doses of reserpine prevented the increase in oxygen consumption produced by parenterally administered thyroxine in rats. De Felice *et al.*(2) observed that reserpine reduced oxygen consumption of euthyroid and hyperthyroid guinea pigs. A direct *in vitro*

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antithyroid effect of reserpine consisting chiefly of inhibition of organic binding of I^{131} has been reported(3). Moncke(4) found that chronic reserpine therapy over a period of months produced "normalization" of basal metabolic rate in a group of 15 hyperthyroid patients. In contrast to the above Goodman *et al.*(5) found that reserpine at the dose levels employed clinically for treatment of hypertension did not affect thyroid function in man; and Ford *et al.*(6) were unable to demonstrate any change in basal metabolic rate or I^{131} uptake of the thyroid gland in euthyroid patients after chronic treatment with a total alkaloid extract of *Rauwolfia serpentina*. Since rats fed highly purified diets are particularly sensitive to thyroid feeding as evidenced by early death, apparently due to cardiac failure(7,8), it was felt that if reserpine has an anti-thyroid effect it might be manifest in terms of an increased survival time in the immature thyroid-fed rat. Findings indicate, however, that rather than prolonging survival as might have been anticipated, reserpine in large doses significantly increased the toxic effects of thyroid feeding.

Procedure. The basal ration consisted of sucrose, 65.8%; casein,† 24%; salt mixture,‡ 5%; corn oil 5%; and l-cystine, 0.2%. To each kg of the above diet were added the following vitamins: thiamine hydrochloride, 20 mg; riboflavin, 20 mg; pyridoxine hydrochloride, 20 mg; calcium pantothenate, 60 mg; nicotinic acid, 100 mg; ascorbic acid, 200 mg; biotin, 4 mg; folic acid, 10 mg; para-aminobenzoic acid, 400 mg; inositol, 800 mg; Vit. B_{12}, 150 μg; 2-methyl-naphthoquinone, 5 mg; choline chloride, 2 g; Vit. A, 5000 U.S.P. units; Vit. D_2, 500 U.S.P. units; and alpha-tocopherol acetate, 100 mg. The vitamins were added in place of an equal amount of sucrose. Male and female rats of the Holtzman strain were selected shortly after weaning and were fed either the basal ration indicated above or the basal ration plus the supplements listed in Table I. Food and water was provided *ad lib.* Animals were fed daily and all food not consumed 24 hours after feeding was discarded. Feeding was continued for 6 weeks or until death whichever occurred sooner. Initial body weight, number of animals per group and results obtained are summarized in Table I.

Results. In agreement with earlier findings(7,8) growth was retarded and rats failed to survive when fed a highly purified ration containing massive doses of thyroid. The effects obtained were proportional to amount of thyroid administered. Reserpine§ when fed with the basal ration at a level of 10 mg/kg of diet had no deleterious effects on either growth or survival; when fed at a level of 20 mg/kg diet, however, it resulted in significant growth retardation although no deaths occurred during the experimental period. Combined supplements of reserpine at a level of 20 mg/kg ration and desiccated thyroid at all levels caused 100% mortality within an experimental period of 10 days. Reserpine when fed at a level of 10 mg/kg ration in conjunction with the 0.5% thyroid supplement resulted in a weight increment significantly less than that obtained with the thyroid supplement alone and also decreased average length of survival. It is apparent from these findings that whereas decreasing doses of thyroid did not significantly affect the deleterious effects of combined reserpine-thyroid supplementation when reserpine was fed at a level of 20 mg/kg ration, decreasing the dosage of reserpine when thyroid was kept at the 0.5% level reduced the severity of the effects obtained. The potentiating effects of reserpine on symptoms of thyrotoxicity were observed both in male rats (Exp. 1) and female rats (Exp. 2).‖

† Vitamin-free Test Casein, General Biochemicals, Chagrin Falls, O.

‡ Hubbel, Mendel and Wakeman Salt Mixture, General Biochemicals, Chagrin Falls, O.

§ Reserpine was kindly provided by Dr. George Cronheim of Riker Labs., Los Angeles, Calif.

‖ Similar studies were conducted with female rats of the Holtzman strain fed a natural food stock ration (ground Rockland Rat Diet, Arcady Farms Milling Co., Chicago, Ill.). No deaths occurred in rats fed the unsupplemented stock ration, the stock ration plus 0.125% desiccated thyroid or the stock ration plus 20 mg reserpine/kg of diet (6 animals/group). All 6 rats fed the stock ration supplemented with above amounts of both thyroid and reserpine, however, died within 12 days.

TABLE I. Effects of Reserpine on Weight Increment and Survival Time of Thyroid-Fed Rats.*

Supplements fed with basal ration	Gain (g) in body wt after following days of feeding 21st	42nd	Mortality, %	Avg survival time of decedents, days
Exp. 1				
None	133.6 (10)	213.6 (10)	0	
.125% thyroid	118.4 (10)	193.9 (10)	0	
.25% "	103.0 (10)	134.2 (3)	70	35.2
.5% "	95.4 (10)	143.4 (1)	90	31.6
10 mg reserpine/kg diet	144.8 (10)	228.6 (10)	0	
20 mg *Idem*	94.2 (10)	170.5 (10)	0	
.125% thyroid & 20 mg reserpine/kg diet			100	8.7
.25% *Idem*			100	8.3
.5% "			100	6.3
.5% thyroid & 10 mg reserpine/kg diet	50.3 (5)		100	21.8
Exp. 2				
None	96.9 (10)	139.2 (10)	0	
.125% thyroid	98.2 (10)	141.2 (10)	0	
20 mg reserpine/kg diet	62.0 (10)	107.2 (10)	0	
.125% thyroid & 20 mg reserpine/kg diet			100	8.6
Exp. 3				
None	98.4 (6)	144.2 (6)	0	
.125% thyroid	98.5 (6)	146.7 (6)	0	
20 mg reserpine/kg diet	83.2 (6)	108.4 (6)	0	
.125% thyroid & 20 mg reserpine/kg diet			100	12.4
200 mg chlorpromazine HCl/kg diet	94.4 (6)	123.9 (6)	0	
.125% thyroid & 200 mg chlorpromazine HCl/kg diet	84.7 (6)	108.6 (6)	0	
2700 mg 2-methyl-2-n-propyl-1,3-propanediol dicarbamate/kg diet	88.2 (6)	117.7 (6)	0	
.125% thyroid & 2700 mg 2-methyl-2-n-propyl-1,3-propanediol dicarbamate/kg diet	93.9 (6)	126.4 (6)	0	

Values in parentheses indicate No. of animals which survived and on which averages are based.

* In Exp. 1 each group consisted of 10 male rats with avg initial body wt of 57.3 to 56.8 g; in Exp. 2 each group consisted of 10 female rats with avg initial body wt of 46.0 to 46.2 g; in Exp. 3 each group contained 6 female rats with avg initial body wt of 56.0 to 56.4 g.

Tests were subsequently conducted to determine the comparative effects of reserpine, chlorpromazine hydrochloride¶ and 2-methyl-2-n-propyl-1, 3-propanediol dicarbamate** on symptoms of thyrotoxicity in the immature thyroid-fed rat. Findings indicate that whereas reserpine when fed at a level of 20 mg/kg of diet resulted in a 100% mortality within 14 days when fed with 0.125% desiccated thyroid, no deaths occurred in any of the rats fed this dose of thyroid in conjunction with chlorpromazine hydrochloride or 2-methyl-2-n-propyl-1, 3-propanediol dicarbamate, nor was weight increment of rats fed the latter drugs in conjunction with desiccated thyroid significantly less than that of rats fed these drugs without the thyroid (Exp. 3, Table I).

No data are available to indicate the mechanisms whereby doses of reserpine and thyroid, which when administered individually are non-lethal, rapidly result in death when administered concurrently. These results were unanticipated since reserpine has been reported to prevent increase in oxygen consumption in rats(1) and guinea pigs(2) administered thyroid hormone. Further-

¶ Thorazine HCl, Smith, Kline & French Labs., Philadelphia, Pa.

** Miltown, Wallace Labs., New Brunswick, N. J.

more, Kuschke and Gruner's(1) suggestion that reserpine functions as a thyroxine antagonist would lead one to expect that rather than potentiating the toxic effects of thyroid administration reserpine would counteract such effects. In this regard it may be of importance that the dosage of reserpine employed in the present experiment was larger than that employed by others. It is possible, however, that the growth retardation and death obtained when thyroid and reserpine were fed concurrently were due not to a potentiation of thyrotoxicity as a result of reserpine administration but rather a potentiation of reserpine toxicity as a consequence of thyroid feeding.

Summary. Doses of reserpine and desiccated thyroid which were non-lethal to immature rats when administered separately resulted in a 100% mortality within a 2 week period when administered concurrently.

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Effect of Polycarboxylic Acids on Blood Clotting.* (24292)

EDWIN E. GORDON (Introduced by H. L. Blumgart)

The Department of Medicine, Harvard Medical School, the Medical Research Department, Yamins Research Laboratories, and the Medical Service, Beth Israel Hospital, Boston, Mass.

The anticoagulant action of citrate has been known for more than 60 years(1). The similarity in molecular structure of isocitrate and aconitate to citrate suggested that these tricarboxylic acids might also interfere with the clotting mechanism. To test this hypothesis, isocitrate, aconitate and the sodium salts of other polycarboxylic acids were incubated with human blood. Of the acids tested, those with 3 free carboxyl groups were effective in prolonging the clotting time.

Methods. Two ml of venous blood from normal volunteers or from patients without known clotting defects was discharged into tubes containing a solution of 0.5 ml of the appropriate test substance and into control tubes charged with 0.5 ml of 0.85% saline. The tubes were stoppered, inverted several times and placed upright in a rack at room temperature. Clotting time was defined as the interval between termination of mixing and formation of a firm clot which remained at the bottom when the tube was completely inverted. The polycarboxylic acids, obtained from commercial sources,† were put into solution, neutralized to pH 7 (pH Hydrion paper) and made up to volume with distilled water or saline just prior to use. The citric acid content of the commercial *cis*-aconitic acid, *trans*-aconitic acid, dl-Na_3 isocitrate, and tricarballylic acid was found to be less than 0.05%

* Supported by a grant from the Massachusetts Heart Assn.

† Citric acid was purchased from Baker Chemical Co.; *cis*-aconitic acid, dl-malic acid, fumaric acid, tricarballylic acid, malonic acid, dl-isocitric acid lactone and Na_3 isocitrate were purchased from the California Foundation for Biochemical Research, Los Angeles. The source of the other compounds tested was the Nutritional Biochemical Corp., Cleveland, Ohio. *Trans*-aconitic acid, recrystallized from the commercial preparation, was found to be 86% pure by chromatography(2).

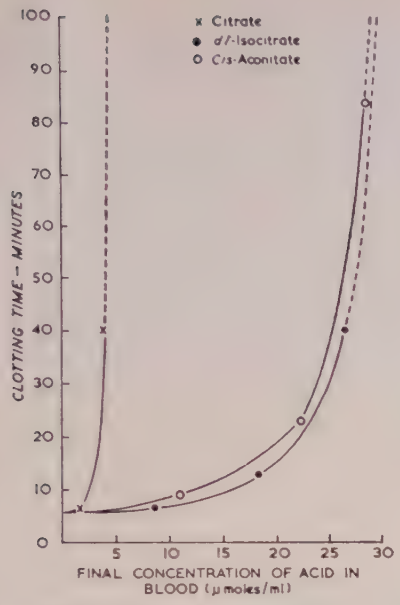

FIG. 1. Effect of varying concentrations of citrate, dl-isocitrate and *cis*-aconitate on clotting time of whole blood. Two ml of blood was added to 0.5 ml of a solution containing the test substance.

as determined by the method of Natelson, Pincus & Lugovoy(3). This amount of citric acid present as a contaminant would not appreciably influence blood clotting time.

Results. Both isocitrate and *cis*-aconitate were found to increase clotting time of whole blood (Fig. 1). The effect was dependent upon the concentration of polycarboxylic acid in the blood. Prolongation of clotting time was identical in the presence of equimolar concentrations of isocitrate and *cis*-aconitate. At a final concentration of 46 μmoles/ml both isocitrate and *cis*-aconitate increased clotting time to over 24 hours (Table I). The unnatural isomer, *trans*-aconitate, prolonged the clotting time, but was less effective than the *cis* isomer. Tricarballylate was also found to influence clot formation. The lactone of isocitric acid was relatively ineffective in increasing the clotting time. Only a slight prolongation of clotting time was noted when each of the dicarboxylic acids, α-ketoglutarate, succinate, fumarate, malate, glutarate, citraconate, glutamate and malonate was added to blood.

In other experiments, 2.0 ml of blood was mixed with 0.5 ml of 0.23 M *cis*-aconitate and permitted to stand for 4 hours. Control tubes contained 0.5 ml of saline and a drop of heparin (1000 U/ml) instead of *cis*-aconitate. At the end of the incubation period the cellular elements were removed by centrifugation, plasma proteins were precipitated with 3 volumes of 20% trichloracetic acid and the citric acid of the supernatant was determined. Plasma that had been incubated with *cis*-aconitate contained 5-7 μg citric acid/ml more than the control. This experiment indicated that a quantity of *cis*-aconitate, insufficient to affect clotting time, had been converted to citrate. Incubation of isocitrate in a similar manner did not reveal conversion of this acid to citrate.

Recalcification times were carried out on plasma from whole blood that had been incubated for 2 hours at room temperature with 0.046 M isocitrate and with *cis*-aconitate. Plasma clots formed between 5 and 10 min-

TABLE I. Effect of Polycarboxylic Acids on Clotting Time of Human Blood. 2 ml of blood mixed with 0.5 ml of 0.23 M acid (neutralized to pH 7). Control tubes contained 2 ml of blood plus 0.5 ml of 0.85% saline. Values are the means of a number (in parentheses) of experiments. Range of values is given beneath mean value in each case.

Acid	Clotting time	
	Control	Sample
Tricarboxylic	Min.	
Citric* (4)	5	>24 hr
Cis-aconitic (4)	5	>24 "
Trans-aconitic (4)	7	5–18 "
Isocitric (4)	6	>24 "
Isocitric lactone (4)	5	30 min.
		13–49 "
Tricarballylic (4)	5	>24 hr
Dicarboxylic	Min.	Min.
α-ketoglutaric (4)	5	10.5
		9–12
Succinic (4)	6	28
		18–41
Fumaric (4)	6	8
		7–9
Malic (7)	5	21
		17–42
Glutaric (4)	6	10.5
		4–15
Citraconic (3)	7	27
		26–29
Glutamic (2)	5.5	10
		8–12
Malonic (5)	5	12
		8–16

* Concentration .115 M.

utes after addition of 0.2 ml of 0.02 M $CaCl_2$ to 0.2 ml of plasma. Clot formation was not apparent 4 hours after $CaCl_2$ had been added to plasma from the tricarballylate incubated blood samples.

Discussion. These experiments indicate that blood clotting is inhibited *in vitro* in the presence of isocitrate and *cis*-aconitate as well as in the presence of citrate. None of the dicarboxylic acids tested was effective in prolonging clotting time; the lactone of isocitric acid was similarly ineffective. The mechanism of the inhibition has not been established. Although other interpretations are possible, it is likely that the tricarboxylic acids chelate with ionic calcium through the 2 terminal carboxyl groups as postulated by Heinz(4) to form relatively undissociable calcium complexes in the blood. Tricarballylate has been shown to complex with ionic calcium in pure solutions(5), but it appears from the recalcification experiments that its ability to prevent clotting stems from interference with the coagulation mechanism other than its Ca^{++} binding ability.

In the normal state it is unlikely that *cis*-aconitate or isocitrate play a significant role in prevention of blood clotting *in vivo* because of the small concentration of these substances in blood(6). It is possible, however, that these intermediates of the Krebs cycle might influence the intracellular concentration of ionized calcium. This concept would be especially important in those tissues, such as nerve and muscle, where small changes in concentration of ionized calcium are known to exert profound effects(7). Conversely, increased concentrations of ionized calcium in tissues might influence citric acid cycle metabolism by complexing with citrate, isocitrate or *cis*-aconitate thereby interfering with enzyme substrate formation.

Summary. Human blood was incubated *in vitro* with the sodium salts of a number of di- and tricarboxylic acids and their effect on clotting time was noted. *Cis*-aconitate, *trans*-aconitate, isocitrate and tricarballylate markedly increased clotting time of whole blood. Prolongation of clotting in the presence of isocitrate and *cis*-aconitate was dependent upon their concentration in blood. The possible mode of action of these metabolically active compounds and the implications of the findings were discussed.

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Role of Relaxin in Stimulating Mammary Gland Growth in Mice.* (24293)

HIROSHI WADA† AND CHARLES W. TURNER
(With technical assistance of Mary E. Powell)
Department of Dairy Husbandry, University of Missouri, Columbia

It has been shown that estrogens (E) will stimulate mammary gland duct growth while E and progesterone (P) in suitable amounts and ratios will stimulate lobule-alveolar growth in normal and gonadectomized male and female animals(1-6). It has been suggested that relaxin synergizes with E and P in stimulating lobule-alveolar growth in rats

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† Research scholar, Ministry of Education of Japanese Government. This investigation supported in part by grant from Am. Cancer Soc.

(7,8) and potentiates estrogen in guinea pigs and rabbits(9), but is of little value in mice (10). Using mammary gland spreading factor as an index of mammary gland growth in rats, relaxin and E stimulated a large increase of spreading factor, but relaxin was of no benefit as an addition to combination of E and P. Relaxin alone failed to stimulate lobule-alveolar growth(11-13). In analyzing the variable observations on the role of relaxin in stimulating mammary lobule-alveolar growth, it was noted that relaxin was most effective when less than optimal amounts of P were administered. In fact, E and relaxin alone seemed to be effective(8,13). These observations could all be harmonized by the theory that injection of E and P stimulates endogenous production of relaxin. Relaxin thus stimulated acts upon the pituitary to stimulate mammogen secretion, resulting in mammary lobule-alveolar growth. If this is true, then E and relaxin should be as effective as E and P in stimulating mammary gland growth. Total desoxyribosenucleic acid (DNA) of the mammary gland, shown to be an improvement in quantitative estimation of lobule-alveolar growth(5,14,15,16) has been used in the present study to substantiate this hypothesis.

Materials and methods. Since relaxin is obtained from sow ovaries, tests were conducted to insure freedom from progesterone contamination in relaxin preparations used. None was found, but all relaxin‡ preparations were routinely washed with ether (anhydrous) several times as a safeguard. Male albino mice (intact and castrated) weighing 16-18 g were used. To their feed was added 1.23 mg/kg of diethylstilbestrol for approximately 4 weeks to stimulate extensive duct system. Suspensions of relaxin alone and relaxin in combination with estradiol benzoate in 0.1 ml of olive oil were then administered subcutaneously daily for 10 days. On 11th day, mammary glands were removed from each mouse killed by excessive ether anesthesia, and ¾ of glands were used for DNA determination(15) and ¼ reserved for examination of whole mounts. After diethylstilbestrol pretreatment, one group of mice was hypophysectomized, then divided into 2 lots, one receiving E alone and the other E and relaxin for 10 days.

Results. In previous work it was shown that total DNA content of male mice glands after 4 weeks of diethylstilbestrol pretreatment to induce duct growth averaged 1.51 mg ± 0.56 mg. Injection of 0.75 μg estradiol benzoate and 0.75 mg progesterone daily for 10 days increased total DNA of the glands to 2.28 mg ± 0.275 mg representing growth of the lobule-alveolar system(15).

When the same level of estradiol benzoate (0.75 μg) was injected with graded levels of relaxin in both normal and castrated male mice, there was an increase in average total DNA (Table I). Optimal level of relaxin appears to be 2.5 guinea pig units (GPU). Injection of E alone failed to stimulate an increase in total DNA in intact animals, but induced a slight increase in total DNA in the castrated animals. When relaxin alone was injected in graded amounts total DNA remained stationary throughout entire dosage range, though it was clearly higher than those injected with olive oil.

Examination of whole mounts of the mammary glands revealed extensive growth of the lobule-alveolar system in mice receiving E and relaxin, whereas those receiving either E or relaxin, but not both, showed only duct growth. The extent of lobule-alveolar growth in various groups was roughly proportional to their DNA content. Intact animals injected with E and 2.5 GPU relaxin showed the best development of lobule-alveolar system, and mammary gland growth of some animals in this group was equivalent to that at 8 days of pregnancy(17).

Castrated mice injected with relaxin alone showed no alveolar growth, whereas those injected with E showed very slight lobule-alveolar growth in some cases. With E and 2.5 GPU relaxin best alveolar growth was observed. While intact animals showed slightly greater total DNA, the possible influence of androgen secretion will require further study.

‡ Relaxin preparation, 1164A-Lot 53, kindly supplied by Dr. R. L. Kroc, Chilcott Lab., Morris Plains, N. J., assayed 30 GPU/mg.

TABLE I. Effect of Estrogen and Relaxin on Mammary Alveolar Growth in the Mouse.

Treatment (amt/day)		No. of mice	Total DNA Mean (mg)	S.E.	DNA/mg D.F.F.T.* Mean (μg)	S.E.
Intact male						
Control		10	1.369	.088	33.5	1.4
Olive oil	.1 ml	9	1.347	.130	31.9	1.5
Olive oil +						
Estradiol benzoate	.75 μg	23	1.572	.047	31.5	1.0
E.B. + relaxin	.62 GPU	11	1.937	.091‡	37.4	1.4
Idem	1.25 "	16	1.999	.119‡	38.6	1.4
"	2.50 "	18	2.258	.095‡	39.3	1.2
"	5.00 "	16	2.019	.161†	39.4	1.9
Olive oil + relaxin	1.25 "	8	1.775	.139§	36.6	2.3
Idem	2.50 "	9	1.713	.132	42.2	2.2
"	5.00 "	32	1.737	.074§	34.8	3.3
"	10.00 "	9	1.626	.076	32.3	1.3
Olive oil + E.B. + P	.75 mg	5	2.278	.275	26.5	5.0‖
Castrate male						
Olive oil +						
Estradiol benzoate	.75 μg	7	1.808	.106	26.9	1.5
E.B. + relaxin	.62 GPU	5	1.787	.078	28.7	1.7
Idem	1.25 "	7	1.797	.158	29.6	1.4
"	2.50 "	6	2.144	.142	31.3	2.2
"	5.00 "	7	1.858	.143	32.0	1.3
Olive oil + relaxin	5.00 "	7	1.645	.072	32.2	.8
Hypophysectomized male						
Olive oil +						
Estradiol benzoate	.75 μg	6	1.477	.089	42.9	4.2
E.B. + relaxin	2.50 GPU	6	1.708	.071	43.2	2.6

* D.F.F.T. = Dry, fat-free tissue. † Significant at 1% level when compared to group inj. estradiol benzoate .75 μg. ‡ Significant at .1% level. § Significant at 5% level when compared to group inj. olive oil alone. ‖ Data from Damm and Turner(15).

Hypophysectomized male mice treated with E showed evidence of slight regression in total DNA. When E and 2.5 GPU of relaxin were administered, total DNA equaled that of E in castrate mice. No lobule-alveolar growth was stimulated in these animals. Hypophysectomized animals injected with combination of E and relaxin were somewhat more vigorous than those injected with E alone in the later half of injection period, and the duct system of the former seemed to be more normal in comparison to involuted (shriveled) condition of the latter.

Discussion. The present study shows that E and relaxin in optimal amounts stimulate growth of the mammary lobule-alveolar system of intact and castrate male mice equal to that obtained by injection of E and P for equal periods(15). Two indices of growth were employed, namely, total DNA of the glands and visual observation of whole mounts to determine type and extent of gland growth. In absence of the pituitary, E and relaxin are without effect upon lobule-alveolar growth. These observations confirm previous reports that ovarian hormone-relaxin combinations are ineffective in hypophysectomized animals (7,8). Since relaxin is not effective in hypophysectomized animals, it is not a substitute for pituitary mammogen.

It has been suggested that P acts upon the anterior pituitary to stimulate increased secretion of mammogen(18). Present data suggest that relaxin may act in this way and thereby stimulate lobule-alveolar growth.

As a tentative theory, it is suggested that normal growth of the lobule-alveolar system during pregnancy is dependent upon the following sequence of hormone action. E and P together stimulate the endogenous secretion of relaxin. Previous work(19) suggested that E alone has a direct local action upon the mammary gland area in increasing hyperemia and permeability of the vascular system,

thereby increasing circulating nutrients and hormones available to the mammary gland.

Relaxin rather than P acts upon the anterior pituitary to stimulate increased secretion of mammogen. This theory would assign P to an indirect role in mammary gland growth although it is possible that P and relaxin are equally effective at the pituitary level.

The hormones and glands involved in endogenous relaxin secretion become of paramount importance if relaxin rather than P stimulates pituitary mammogen secretion and lobule alveolar growth. Since the growth of the mammary gland of male animals is stimulated by E and P, it would follow that relaxin may be secreted in the male although, to date, relaxin has not been detected in male animal blood following injection of E and P.

Summary. 1) Using DNA as index of mammary gland lobule-alveolar growth in intact and castrate male mice, it was shown that daily injection of 0.75 μg estradiol benzoate and 2.5 GPU of relaxin for 10 days stimulated increases in DNA equal to that produced by the same level of estrogen and 0.75 mg progesterone. Therefore, relaxin no longer need be considered merely a synergist of estrogen and progesterone in mammary gland growth. 2) Estrogen and relaxin at above levels were without effect upon mammary alveolar growth in hypophysectomized mice. Relaxin, therefore, is not comparable to pituitary mammogen as a stimulator of mammary gland growth. 3) It is suggested estrogen and progesterone stimulate endogenous secretion of relaxin and relaxin, in turn, stimulates increased secretion of mammogen by the pituitary.

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Surface Activation of Plasma Clotting: a Function of Hageman Factor.* (24294)

CHARLES L. JOHNSTON, JR., JOHN H. FERGUSON AND FRIEDA A. O'HANLON
Department of Physiology, University of North Carolina, Chapel Hill

The influence of surface contact on blood clotting has been recognized since the observations of Hewson (1770), Lister (1863), and Freund (1886). Modern literature contains many experiments which have attempted to implicate one or other of the clotting factors. Previously, no one explanation of the effect seemed adequate. Recent evidence suggests that Hageman factor may have a role in surface phenomena(1,2,3). In this communica-

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tion, experimental data are presented on 4 new cases of Hageman trait and other factor deficient plasmas, their reaction on exposure to asbestos surface, and a comparison of the effect of addition of 'purified' Hageman factor to normal and deficient plasmas. The results point to a key role of the Hageman factor in surface effects on blood clotting and show that other clotting factors are not involved.

Materials and methods. Normal (N) plasmas were used as fresh, *aged* (AcG-, *i.e.* proaccelerin poor), or $BaSO_4$ fractions ('supernate' and 'eluate'). Preserved (lyophilized or frozen at –20°C) plasmas from patients with clotting factor deficiencies were obtained from our diagnostic laboratory, directed by Dr. John B. Graham, operated jointly by Pathology and Physiology departments. These comprised a test panel of plasmas and included Hageman deficient (Hag.-), hemophilic (AHF-), Christmas disease (PTC-), hypoproconvertinemic (Proc.-), and Stuart deficient (Stuart-). The diagnosis of Hageman trait was confirmed by cross match testing of our first patient's (L.C.) plasma with that of the original Hageman, provided by Dr. O. D. Ratnoff. Ratnoff's technics (3, personal communication) were followed in efforts to prepare 'purified' Hageman factor from sera of (A) recalcified, normal, outdated bank blood, (N) fresh normal, and (H and I) 2 Hageman trait cases, A.M. and J.I.† Asbestos powder was prepared and used as follows: asbestos (Baker's medium-fiber, acid washed) was powdered under water in a Waring Blendor, water removed by suction filtration, and residue dried at 200°F. After drying, clumps were reduced with mortar and pestle. The product was a reasonably uniform, grayish-white powder. Thirty mg were added to 1 ml of various plasmas, and agitated at intervals for 30 minutes at room temperature. The supernate, recovered by centrifugation, was designated *treated* or *activated*. Preliminary tests showed no further effects on increasing either amount of asbestos or contact time. Several *clotting tests,* some with modification, were used to see if, independent of their original purpose, they could serve the needs of our inquiry. These tests, one-stage systems, timed clotting at 37°C on addition of a thromboplastic agent and calcium to test plasma (or mixture). The prothrombin time (PT) test using 0.1 ml plasma, 0.1 ml thromboplastin and 0.1 ml 0.02 M $CaCl_2$ was the prototype. Plasma mixtures with appropriate factor-free substrates were used in specific assays(4) for prothrombin, proaccelerin, or proconvertin and/or Stuart factor. We used a saline extract of acetone dried human brain powder as thromboplastin. When cephalin, obtained by chloroform extraction of the human brain powder, was substituted for thromboplastin, the PT was converted into the partial thromboplastin time test (PTT)(5) and the standard proconvertin assay into a 'modified' proconvertin assay. The Stypven time used Russell's viper venom as the thromboplastic agent. In the new uses, the test system terminology was for identification and no longer had the original significance.

TABLE I. Asbestos Treatment—Normal Plasma. 7 test systems. Clotting-times, sec., 37°C.

Test system	Untreated	Treated
Partial thromboplastin time (PTT)	75.5	60
Prothrombin time (PT)	11.7	10.7
Standard proconvertin	18.5	13.8
Modified "	107.0	66.4
Stypven time	16.8	17.3
Specific one-stage prothrombin	22.9	22.7
" " proaccelerin	25.0	26.3

Results. Asbestos activation. Table I shows representative results obtained when normal human plasma was exposed to powdered asbestos. Clotting-times shorter than controls were obtained in 4 of 7 test systems. No significant alteration was noted in 3 assays. Similar results were obtained with bovine plasma and oxalate or citrate as anticoagulant. *Shortening of test clotting-times will be referred to as plasma activation by surface action of asbestos.*

The plasma panel was tested for asbestos activation by the standard and modified proconvertin assays. Table II shows that no significant change in lyophilized Hag.-

† Blood from this patient was made available through the courtesy of Dr. A. I. Chernoff, Vet. Adm. Hosp., Durham, N. C.

TABLE II. Asbestos Treatment—Various Plasmas. Clotting-times, sec., 37°C, in 2 types of test, (a) before and (b) after asbestos treatment of plasmas.

Plasma tested	Standard proconvertin test (a) Untreated	Standard proconvertin test (b) Treated	Modified proconvertin test (a) Untreated	Modified proconvertin test (b) Treated
Normal	18.5	13.8	107.0	66.4
Hag.-	*24.4*	*24.2*	*135.7*	*135.5*
AHF-	16.6	14.9	108.4	85.4
PTC-	22.0	19.4	98.8	70.9
AcG-	16.6	15.4	101.0	87.4
Stuart-	97.9	73.9	183.0	143.5
Proc.-	113.9	88.6	130.0	92.1

plasma resulted from such treatment. However, all other plasmas showed some degree of shortening (acceleration) of post-treatment clotting-times.

The response of normal $BaSO_4$ fractions to asbestos was studied. These fractions and Hag.- were treated separately, then mixed, assayed by the 2 proconvertin tests, and compared with untreated mixtures. The eluate was not activated when tested either alone or mixed with Hag.-. $BaSO_4$ treatment removed proconvertin and Stuart factor to which these assays were sensitive; hence, the supernate alone did not give measurable clotting-times. However, the mixture of treated supernate and Hag.- gave times *shorter* than the untreated control. This shortening resulted from treatment of the supernate, since Hag.- alone (Table II) was not activated. These data indicated that some plasma factor, present in normal plasma, not adsorbed by $BaSO_4$, and lacking in Hag.- was responsible for surface activation. We have been unable to confirm that a "small amount" of Hageman factor may be adsorbed(3).

Table III shows PTT data for control substrate clotting-times and equal volume mixtures of substrate and untreated test plasmas. Asbestos treated normal plasma shortened clotting-times more than untreated normal when mixed with *all* substrate plasmas. Treated Hag.- did not additionally shorten *any* substrate clotting-time below pretreatment levels. Indeed, in some instances there was lengthening instead of shortening; this is being investigated. Minor secondary clotting factor deficiencies are represented in Table III by failure of untreated normal to correct completely deficiencies in substrate plasmas. These do not preclude reaching the significant conclusion that Hageman factor deficient plasma fails to be activated by asbestos.

To show that the noted phenomena were unrelated to storage and manipulation of panel plasmas, fresh normal, AHF-, and Hag.- plasmas were obtained and tested immediately. Prothrombin time (PT) and partial thromboplastin time (PTT) test were used. There was failure of Hag.- to be activated: PT 12.1″ before, 12.2″ after treatment and PTT 286″ before, 319″ after treatment. The other 2 samples showed activation: AHF-: PT 12.3″ before, 10.6″ after treatment and PTT 216″ before, 157″ after treatment; normal: PT 12.4″ before, 8.8″ after treatment and PTT 92″ before, 60″ after treatment. Thus, freshly obtained materials reacted to asbestos in the same manner as the stored, and the noted minor secondary deficiencies were not part of the phenomenon. Since PT data on stored plasmas were of questionable significance, these fresh plasmas gave the best evidence that surface activation influenced the PT test. Only minor differences were noted when silicone was used to minimize initial surface effects.

Effects of purified Hageman factor prepa-

TABLE III. Effect of Asbestos Treatment on Normal and Hageman Deficient Plasmas: PTT Controls for Substrate Plasmas, and Mixtures of Equal Parts Test and Substrate Plasmas, before and after Treatment. Clotting-times, sec., 37°C.

Test plasma	Substrate plasma: Normal	AHF-	PTC-	Hag.-	Stuart-	Proc.-	AcG-	$BaSO_4$ supernate
Substrate, untreated	75.5	212	488	860	291	115.5	442	
Normal, "	75.5	80.2	172	118.2	107.6	124.2	87.2	91.4
Normal, treated	60.0	74.2	102	100	90.8	90.6	70.0	73.0
Hag.-, untreated	122.4	89.6	212	860	210.8	157.0	136.7	140.7
Hag.-, treated	125.0	91.5	434	825	241.6	172	140.8	150.0

TABLE IV. Tests of "Purified" Hageman Factor Preparation (N) and (H): Results of Addition to Normal and Hag.- Plasmas. Clotting-times, sec., 37°C.

Test material .09 cc		.01 cc	PTT
Oxalated normal	+	Barb.-sal. buffer	79.8
		N (1:1)	39.0
		H (")	84.3
		N (1:10)	60.4
		H (")	91.8
Hag.- (L.C.)	+	Barb.-sal. buffer	402
		Oxalated normal	140.0
		N (1:1)	91.4
		H (")	313

rations. Addition, in 10% volume, of varying dilutions of purified preparation (A) to normal plasma resulted in PTT shortening, which was marked on addition of undiluted (1:1) material, and demonstrable at 1:100 but not 1:200 dilution. The normal plasma presumably had adequate Hageman factor, so that these results indicated: a) a highly potent concentrate, and b) no evidence of optimum for Hageman factor.

Table IV compared addition of purified preparations (N) and (H) to normal and Hag.- plasmas. (N) markedly accelerated both. However, (H) was ineffective when added to normal and had only minimal effect on the Hag.-, despite the fact that the anticipated concentration of *any* Hageman factor present should have been 100x (see above). When compared with addition of an equivalent volume of normal plasma, (H) contained only a trace of Hageman factor. Similar data were obtained with (I).

Table V gives additional evidence that (H) was inactive, since it failed to shorten substrate plasma clotting-times. Comparably, (N) shortened clotting-times of all substrate plasmas. Another important conclusion is that purified preparations did not contain factors other than the Hageman factor, since no shortening was noted in *any* of the deficient substrates. Thus, similarity of action of the purified preparation to asbestos activated plasma (Table III, V), must be due to the presence or absence of Hageman factor.

Discussion. Asbestos treatment produced "activation" (clotting-times shorter than controls in several test systems) in all plasmas or fractions tested except those lacking Hageman factor. Since 'purified' Hageman factor preparations from normals, *but not Hageman trait patients,* mimicked this clotting-time shortening, it is concluded that Hageman factor is responsible for the phenomenon.

These data permit a new look at previously published work suggesting that other clotting factors are responsible for surface activation of plasma. Alexander(6) summarized the platelet role . . . "Precisely which clotting factor (or factors) is directly influenced by surface is far from clear. Contrary to earlier opinion, it appears that it is not the platelets." Indeed our earlier work showed surface effects in completely platelet-free plasma (7). Those clotting factors most usually held responsible for surface activation of plasma were PTC and proconvertin. Our data showed surface activity in plasma lacking these factors, but containing Hageman factor. Similarly, others(1,2) showed that plasmas lacking either or both PTC and proconvertin had surface activity. Shafrir and de Vries(2) showed activity in plasma devoid of AHF, PTC, prothrombin, proconvertin, and fibrinogen. We confirmed and extended this

TABLE V. Tests of "Purified" Hageman Factor Preparations (N) and (H): Partial Thromboplastin Testing of .01 cc Test Material and .09 cc Plasma Substrate. Clotting times, sec., 37°C.

Test material	Substrate Normal	AHF-	PTC-	Hag.-	Stuart-	Proc.-
Barb.-sal. buffer	85.2	156.0	253	234.0	342.6	129.0
Normal plasma	82.2	102.8	159	178.6	159.2	115.4
N (1:10)	69.1	135.4	234	131.4	307.0	125.4
H (")	81.2	156.0	276	253.3	334.2	132.7
N (1:1)	44.2	91.8	179	75.8	182.0	79.6
H (")	81.1					131.8

latter finding and showed in addition that, as implied by Ratnoff and Margolius(3), surface activation is activation of Hageman factor. Finally, by use of purified Hageman factor devoid of other clotting factors, we mimicked the clot acceleratory activity of asbestos treatment. Comparable preparations from Hageman trait patients were inactive.

Removal of an inhibitor must be considered as an alternative explanation of our data. Shafrir and de Vries(2) believed that an inhibitor was not adsorbed onto glass. We did not feel that asbestos removed an inhibitor, since: 1) treated Hageman deficient plasma did not shorten clotting-times of plasmas containing Hageman factor (Table III); 2) asbestos treatment was mimicked by positive factor addition (Tables III, V); 3) there may have been *increased* inhibition which resulted from asbestos treatment (Table III).

Thus, surface contact may be said to result in Hageman factor activation. In its absence, no activation is noted. The failure of asbestos to activate plasma lacking Hageman factor suggests a *presumptive* test for Hageman trait diagnosis.

Summary. 1. Asbestos treatment of plasma produced activation (clotting-time acceleration) in normal and AHF, PTC, proconvertin, prothrombin, proaccelerin and Stuart deficient plasmas. Hageman deficient plasma was not activated by comparable treatment. 2. Purified Hageman factor preparations from normal sera simulated activation, whereas preparations from sera of Hageman trait patients were inactive in the same test system. 3. The phenomenon of surface activation resulted from Hageman factor activation and was not due to inhibitor adsorption. 4. Asbestos treatment of plasma may be used as a *presumptive* test for Hageman trait.

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Metabolism of Sr^{90} in Adult Beagle Dogs.* (24295)

Betsy J. Stover and David R. Atherton (Introduced by T. F. Dougherty)
Radiobiology Laboratory, University of Utah, Salt Lake City

This study of Sr^{90} metabolism is part of a large program to compare the chronic toxicities of Ra^{226}, Pu^{239}, Ra^{228}(MsTh), Th^{228} (RdTh), and Sr^{90} in young adult beagle dogs. The experimental design, especially relationship of dose levels to maximum permissible and natural levels in man, has been described (1). The basic plan is to compare the effects of these radioactive isotopes in a larger, longer-lived animal (the dog) than is usually used in the laboratory; then to use this information in conjunction with the knowledge of radium poisoning in humans to reappraise maximum permissible levels for other isotopes. To correlate the biological effects with radiation dose, a detailed knowledge of the metabolism of each radioisotope is needed.

Methods. Sr^{90}, with its daughter Y^{90} in equilibrium, in a citrate buffer solution of pH = 3.5, was given in single intravenous injection. Injected doses ranged from 0.56 to 100 μc/kg; duplicate doses served as injection standards. The beagle dogs, healthy young adults 16 to 17 months old, during the excretion study were housed in stainless steel metabolism cages, with perforated floors which permit urine to drain into plastic bottles so that

* Supported by the U. S. Atomic Energy Comm.

there is good separation of urine and feces. Urines were wet ashed with HNO_3; feces were incinerated in a muffle furnace, then digested with HNO_3. At a time to insure Sr^{90} – Y^{90} equilibrium, 1 ml aliquots of ashed excreta samples were measured using a well-type plastic scintillator to detect the beta particles. There are 2 important factors to consider in using this beta detector, which is similar to that described by Hine and coworkers(2). The first is geometry, for detector response decreases as source is moved from bottom to top of the well: *i.e.*, count rate of 3 ml of a given solution is less than 3 times the count rate of 1 ml of the same solution. Fortunately, the polyethylene tubes used are quite uniform (0.16 cm wall, 0.82 cm inside diameter), and the geometry problem is solved by using a constant volume. The second factor is self-absorption. The Sr^{90} standards are dilute solutions of essentially unit density (density of plasma is also approximately one), but densities of ashed excreta samples are greater and the effect is significant. Polyethylene tubes and liner of scintillator well are sufficiently thick to stop all Sr^{90} betas, so we are actually measuring Y^{90}. Detection efficiency for 1 ml sample of unit density is about 0.14 count/Y^{90} disintegration. Increasing density to 1.5 g/ml decreases efficiency to 0.10 count/Y^{90} disintegration, so that a self-absorption correction must be made. If absorption of the betas is represented by an exponential law, the effect of density on detection efficiency, E, is given approximately by $E = c_1/d\ (1 - e^{-c_2 d})$ (eq. 1), where d is density and c_1 and c_2 are constants. Expanding the exponential gives the following approximate but adequate expression, $E = c_3 - c_4 d$ (eq. 2). To determine c_3 and c_4 experimentally, quadruplicate samples were prepared which contained constant amounts of Sr^{90} and Y^{90} in constant volumes of solutions of varying density, and count rates were determined. The resulting equation, arbitrarily setting $E = 1$ when $d = 1$, is $E = 1.59 - 0.59\ d$ (eq. 3). Blood for the plasma concentration study was heparinized. Soon after injection the Sr^{90} in 1 ml of plasma could be determined directly. As plasma concentration decreased, it became necessary to concentrate the Sr^{90} from a larger volume of plasma by co-precipitation with CaC_2O_4.

TABLE I. Twenty-Two Days Cumulative Sr^{90} Excretion.

Inj. dose (μc/kg)	Inj. age (mo)	Fecal (% dose)	Urinary (% dose)	Total (% dose)	$\Sigma F/\Sigma U$
.593	16	30.3	27.3	57.6	1.11
1.74	"	30.2	29.9	60.1	1.01
3.49	"	32.2	26.6	58.8	1.21
11.2	17	41.1	26.3	67.4	1.56
33.2	"	34.3	20.7	55.0	1.65
105.	"	39.6	24.7	64.3	1.60
Avg	16.5	34.6	25.9	60.5	1.34

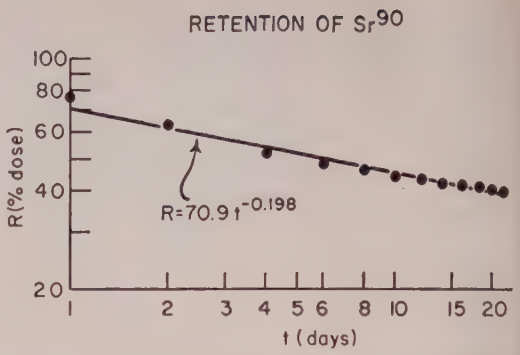

FIG. 1. Retention of Sr^{90} by young adult beagles following a single intrav. inj.

Results. Excretion. Results for each dog are summarized in Table I. Urinary and fecal excretion are nearly equal; average total fecal excretion is 1.34 times average total urinary excretion, and average of individual fecal to urinary ratios is 1.48. There is no apparent dose level effect.

Since this is a continuous excretion study of short duration, the most meaningful presentation of excretion measurements is to calculate retention from cumulative excretion. This has been done for $t = 1$ through $t = 22$ days, and the resulting retention data (Fig. 1)† can be described by a power function. The following equation for retention, R, (% dose) was obtained by applying the method of least squares to logarithms of the data,‡ (t in days), $R = 70.9\ t^{-0.198}$ (eq. 4).

† Indication of a slightly greater slope from day 1 to 4 undoubtedly results from the inherent lag in fecal excretion, which has a significant effect only during initial high excretion period.

‡ Subsequent equations were calculated by the same method.

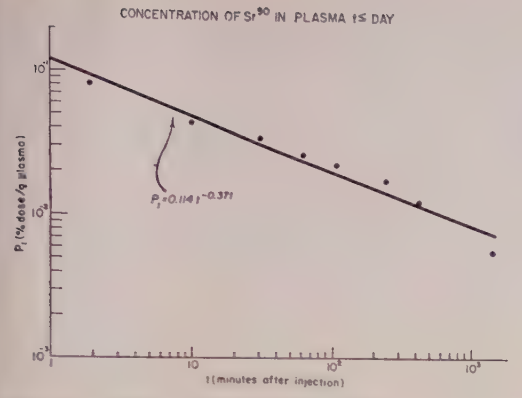

FIG. 2. Plasma concentration of Sr^{90} during first day following inj.

Plasma concentration. Following intravenous injection of soluble Sr^{90}, plasma concentration, as shown in Fig. 2 and 3, decreases rapidly during the first day and continues to decrease through 2 years after injection. To facilitate mathematical treatment, the first day's data and those from one day on were treated separately. Each set can be described by a power function as follows:

$P_1 = 0.114\ t_1^{-0.371}$, $t \leq 1$ day, (eq. 5)
$P_2 = 5.03 \times 10^{-3}\ t_2^{-1.25}$, $t \geq 1$ day, (eq. 6)

where P_1 and P_2 are % dose/g plasma, t_1 is minutes and t_2 is days after injection. From these equations and the average total plasma volume of 450 ml,(3) the per cent dose circulating at any time t after injection can be calculated.

Discussion. Norris and coworkers(4) have used a power function to describe retention of alkaline earth radium in man, and many other studies, (*e.g.*,(1)), have demonstrated the general usefulness of this treatment for retention of alkaline earths in various species. The derivative with respect to time of retention function should be equal to excretion rate and proportional to plasma concentration. Comparing exponents of equations(4) and (6) we observe the expected relationship.§ Since plasma measurements extend over a longer period, the agreement between plasma and retention functions shows that the retention function can be extrapolated without introducing serious error. As further verification, C. W. Mays(5) of this laboratory has determined Sr^{90} retention directly by measuring bremsstrahlung. His data extend to 3 years after injection and his result is very similar to equation(4).

An extensive study of radium metabolism in young adult beagle dogs has also been made at this laboratory(1). Since both groups of dogs were essentially the same age at injection, live in the same environment, and have about the same diet, a reliable metabolic comparison of Sr^{90} and Ra^{226} can be made. Retention, plasma, and excretion results are summarized in Table II. There is a significant difference in retention. Retention of Sr^{90} is slightly less than that of Ra^{226}, but, most interestingly, both decrease at the same rate. During the first day plasma strontium decreases less rapidly than does radium, at one day strontium is about twice that of radium, and thereafter they decrease at the same rate maintaining a ratio of two. Although the fecal to urinary excretion ratio is greater for

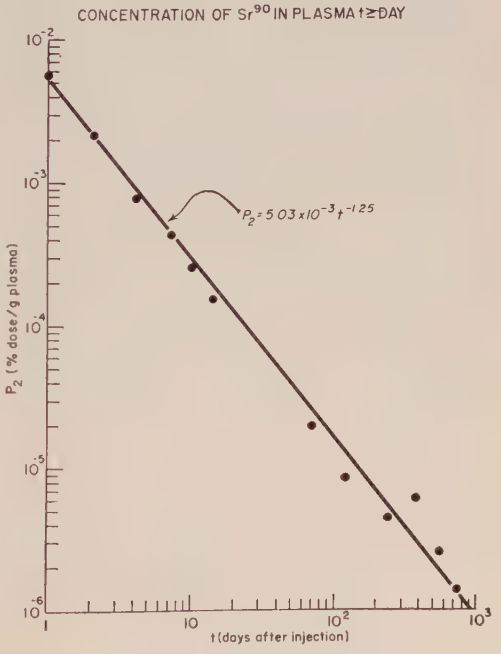

FIG. 3. Plasma concentration of Sr^{90} from 1 day through 2 yr after inj.

§ Actually, plasma concentration decreases a little more rapidly than expected from the derivative of equation(4), which suggests that some of the late plasma values are low.

TABLE II. Comparison of Sr^{90} and Ra^{226}.

Measurement	Sr^{90}	Ra^{226}	Units
Retention	$71\ t^{-.20}$	$79\ t^{-.20}$	% dose, days
First day plasma concentration	$.114\ t^{-.37}$	$.092\ t^{-.45}$	% dose/g, min.
Later plasma concentration	$5.0 \times 10^{-3} t^{-1.25}$	$2.6 \times 10^{-3} t^{-1.24}$	% dose/g, days
Fecal to urinary excretion ratio	1.4	2.5	

radium, the beagle kidney actually excretes radium more effectively than strontium. Renal clearances calculated from data of Table II are 1180 g plasma/day for Sr^{90} and 1690 for Ra^{226}. Similarly, we calculate that daily Sr^{90} excretion is 6.5 times the amount in plasma, while 13.5 times the plasma Ra^{226} is excreted. Thus, excretory mechanisms function more effectively for radium, yet radium retention is higher. This results of course from the higher Sr^{90} plasma concentrations, especially during the period soon after injection, when excretion rate is highest. Although Sr^{90} is not excreted quite as effectively, more is available to be excreted with the net effect that more Sr^{90} than Ra^{226} is excreted.

Higher Sr^{90} plasma concentrations can result from several factors. Less efficient excretion is one, but from the above discussion it cannot be the principal one or strontium retention would be higher. A second possible factor is that a larger fraction of plasma Sr^{90} exists as complexes with proteins and the various anions. (Since strontium ion is smaller than radium ion, it should form stronger complexes.) Our preliminary results, using a double tracer technic indicate that a significantly smaller fraction of plasma Sr^{90} is diffusible. The third possibility is greater re-exchange of Sr^{90} from bone to body fluids. These 3 factors, excretion efficiency, protein complexing, and extent of re-exchange, reflect the chemical difference between radium and strontium and most probably account for the observed metabolic differences.

Although in the above discussion we have emphasized the differences, the 2 isotopes actually are very similar metabolically. Both are chemically like calcium and deposit almost exclusively in the skeleton; the retention equations show the same dependence on time as do the late plasma equations. Since we have observed chemically related differences, the common time dependence leads us to speculate that the principal factor affecting time dependence is the overall skeletal metabolism (exchange and remodelling). Small chemical differences affect details of the metabolism, while normal skeletal processes involving calcium are controlling factors in the main aspects of metabolism of these 2 isotopes. If this is so, then similar studies with calcium and barium should give power functions with the same exponents but different coefficients.

Since we have shown previously(6) that Y^{90} produced *in vivo* from decay of Sr^{90} in the skeleton does not translocate to any significant extent, the retention equation(4) can be used to calculate the average dose rate to the skeleton and other tissues within the range of the beta particles.

Summary. 1. Retention and plasma concentration of Sr^{90} in young adult beagle dogs have been determined for 2 years following a single intravenous injection. Retention (% dose) follows the power function (t in days) $R = 70.9\ t^{-0.198}$. 2. Comparison of these results with those from similar studies with Ra^{226} shows the anticipated similarity in metabolism of Sr^{90} and Ra^{226}. However, there are real differences. Sr^{90} retention is less than that of Ra^{226} even though Ra^{226} is excreted more efficiently. This results from higher Sr^{90} plasma concentration. 3. Since Sr^{90} deposits mainly in the skeleton and its daughter Y^{90} does not translocate, this retention equation can be used to calculate average skeletal dose rates for these dogs.

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The Inactivation of Newcastle Disease Virus Hemolysin by Antiserum and High-Energy Electrons.* (24296)

DWIGHT E. WILSON (Introduced by Robert R. Wagner)
Department of Biology, Rensselaer Polytechnic Institute, Troy, N. Y.

Previous studies on the hemolysin of Newcastle Disease Virus (NDV) have shown that this property is influenced by the physical treatment of the virus(1), is inhibited by calcium ions(2), and is dependent on pH and the temperature of incubation(3). Radiation studies on NDV(4) have shown that there are about 15 hemolysin units per virus and that the form of these units is probably a flat plate 100-140 Å in radius and 20 Å thick. The experiments described here are concerned with the mechanism of action of the NDV hemolysin. The results of Dulbecco *et al.* (5) and Rubin and Franklin(6) indicate that study of virus neutralization by antibody may yield valuable information on structure and components of the virus. Inhibition of the hemolysin by antiserum and inactivation by high-energy electrons were studied.

Methods. The 87909 strain of NDV was used throughout these studies. High titer virus stocks were prepared by inoculating fertile chicken eggs on the tenth day of incubation with about 10^7 NDV particles. After 30 hours of incubation the infected eggs were chilled to 4°C and the allantoic fluid was harvested. Infected allantoic fluid was then centrifuged for 30 minutes at 30,000 g in a Servall SS-1 centrifuge at 4°C. The virus deposit was resuspended in isotonic saline containing 2% sodium citrate and stored at 4°C until used. *Virus assays.* In the hemagglutinin assay, serial 2-fold dilution of virus in citrate saline were added to .2 ml of a 4% suspension of washed chicken red blood cells (rbc). The highest dilution of virus showing agglutination was scored as the endpoint. All virus samples to be used for hemolysin assays were first dried in citrate saline and resuspended in the same medium. This drying is similar in effect to freezing and thawing and increased the hemolysin titer of the virus preparations about 50-fold. Hemolysin titrations were performed in two ways. The *total hemolysis assay* was adapted from Granoff and Henle(1). 1.0 ml of a virus suspension was placed in a test tube with 1.0 ml of washed rbc in citrate saline (pH 7.2). This mixture was incubated at 37°C for one hour, then 1.0 ml of cold phosphate buffered saline (pbs) was added to stop the reaction. Rbc were removed by centrifugation and optical density of the supernatant fluid was measured at 540 mμ. One tube containing rbc but not virus was used to balance the spectrophotometer and thus eliminate the effect of spontaneous hemolysis. In another tube distilled water was added to give 100% hemolysis. The percent hemolysis for each sample was then calculated from the optical densities. The *hemolysis rate assay* is a modification of the total hemolysis assay. 1.0 ml aliquots of a virus suspension were mixed with 1.0 ml of a 1% suspension of washed rbc and incubated at 37°C. The hemolysis reaction was allowed to proceed in different tubes for 1, 2, and 3 minutes, then stopped by addition of 2 ml of cold pbs. Rbc were removed by centrifugation and optical density of the supernatant fluid was measured at 540 mμ. Rate of he-

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TABLE I. Total Hemolysis Assay.

Relative virus concentration	Optical density	% hemolysis
1.0	.420	100%
.8	.400	96
.6	.370	88
.4	.322	77
.2	.240	57
.1	.188	45
.08	.160	38
.06	.138	34
.04	.107	26
.02	.078	19
.01	.046	11
Distilled water	.420	100

Virus preparation contained about 1000 hemagglutinin units/ml before dilution.

molysis was determined by plotting percent hemolysis at each time and calculating percent hemolysis per minute.

Neutralization by antiserum. 1.0 ml of a diluted chicken antiserum against NDV (containing about 400 hemagglutinin inhibiting serum equivalents/ml) was added to 1.0 ml of a virus suspension and incubated for one hour at 37°C. Aliquots of this mixture were then tested for hemagglutinin and hemolytic activity by the methods described above.

Electron irradiation. Virus samples for irradiation were prepared by pipetting .05 ml of high-titer virus on ½ inch diameter round glass cover-slips. These samples were then dried in a vacuum desiccator. Dry samples were irradiated in vacuum with various doses of 1.0 mev electrons which were produced by a High Voltage Engineering Van de Graaff generator. Electron beam currents were measured with a microammeter and electron beam areas were determined by irradiating a piece of osilid paper placed in the sample position and measuring the exposed area of the paper. From the known beam area, beam current, and duration of irradiation, the dose given each sample was calculated in electrons/cm^2. Irradiated samples were resuspended in citrate saline and assayed as described above.

Results. Table I gives data for the total hemolysis assay. Percent hemolysis rises with increasing virus concentration and approaches a maximum of 100% hemolysis at a virus concentration of about 1000 hemagglutinin units/ml. This result is similar to that obtained by Granoff and Henle(1), but differs from that obtained by Sagik and Levine(3), who found that percent hemolysis decreased with increasing virus concentration at pH 5.9-7.7. The latter result was probably due to the fact that their virus preparation contained sufficient calcium ion to inhibit the hemolysis process. A plot of the data from Table I was used as a standard curve for total hemolysis assay.

The percent hemolysis for a given virus concentration increases linearly with time for the first 5 minutes of incubation, then begins to level off. The slope of the straight line portion of this curve is proportional to the virus concentration over a wide range of values (Table II). In the hemolysis rate assay, the percent hemolysis per minute was measured and a plot of the data of Table II was used as a standard curve for the assay.

Fig. 1 shows the effect of antiserum on hemagglutinin and hemolysin of NDV. The percent of active virus hemagglutinin or hemolysin remaining after incubation with antiserum is plotted *vs.* the antiserum concentration in serum equivalents. One serum equivalent is the antiserum concentration which reduces the virus titer to 37% of the controls (5) and corresponds to a concentration of one antibody molecule per virus particle, assuming neutralization to be a monomolecular reaction. Both of these curves show the same rate of neutralization and are "single hit," *i.e.* 1 antibody molecule is sufficient to inactivate either property.

Inactivation of the NDV hemolysin with 1.0 mev electrons using the hemolysis rate assay is shown in Fig. 2. The percent hemolytic activity is plotted *vs.* the electron dose. This single hit inactivation curve may be represented by the equation

TABLE II. Hemolysis Rate Assay.

Relative virus concentration	% hemolysis at 1 min.	2 min.	3 min.	% hemolysis/min.
100	19	37	51	19
84	15	25	46	15
67	10	24	30	11.3
50	8	20	30	11
33	6	11	16	6
17	2.8	4	6	2.3

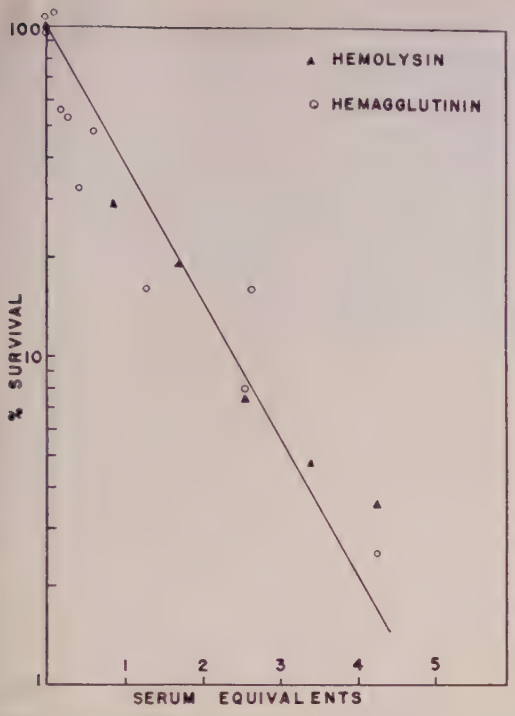

FIG. 1. Inhibition of NDV hemagglutinin and hemolysin by antiserum.

$$N/N_0 = e^{-VD}$$

where N/N_0 is the surviving fraction after an electron dose D, and V is the radiation sensitive volume of the hemolysin unit. The inactivation volume found is 4×10^{-19} cm^3 which corresponds to a molecular weight of 310,000 for the hemolysin unit. For details of the calculation of molecular weights from radiation data see Pollard *et al.*(7).

Inactivation of the NDV hemolysin by 1.0 mev electrons using the total hemolysis assay is shown in Fig. 3. The multiple hit inactivation curve may be represented by the equation

$$N/N_0 = 1 - (1 - e^{-VD})^M$$

where M is the number of hemolysin units per virus. From the slope of the curve in Fig. 3,

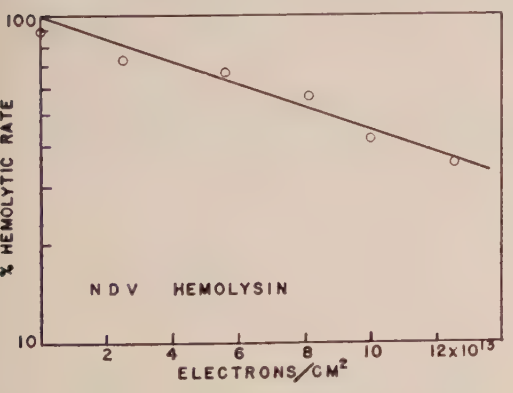

FIG. 2. Inactivation of NDV hemolysin by 1.0 mev electrons using hemolysis rate assay.

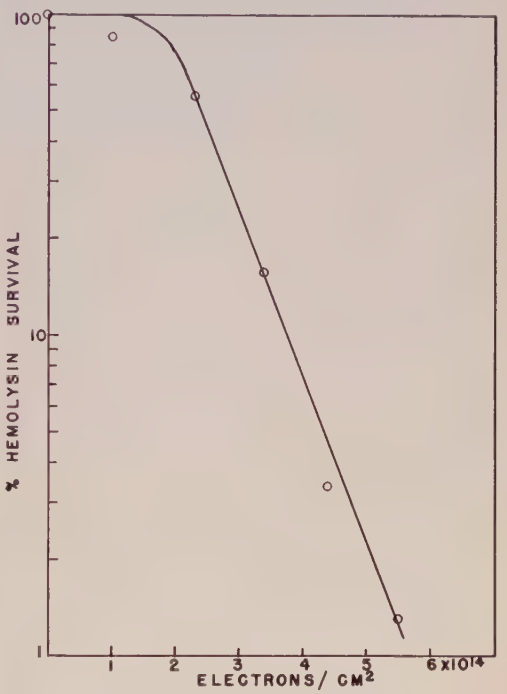

FIG. 3. Inactivation of NDV hemolysin by 1.0 mev electrons using total hemolysis assay.

$V = 4.8(10)^{-19}$ cm^3 which corresponds to a molecular weight of 370,000, and average value of multiplicity in these experiments was 14 ± 5. A similar result was obtained in previous studies on the NDV hemolysin(4) which indicated that there are 15 ± 3 hemolysin units per virus.

Discussion. 1.0 mev electrons produce ionizations along their paths about every 4000 Å. When an ionization occurs inside a hemolysin unit on the virus, chemical bonds in the molecule or molecules making up this unit are broken and the hemolysin unit is no longer able to function. From the shape of the curve in Fig. 3 it can be seen that electron inactivation of one or 2 hemolysin units does not destroy hemolytic activity, but that all 15 units

must be inactivated before this activity is lost. Thus only one intact hemolysin unit per virus particle is necessary for the virus to be able to lyse rbc.

In Fig. 2, reduction of rate of hemolysis by NDV is seen to be proportional to dose of radiation and is single hit. Rate of hemolysis depends on number of intact hemolysin units per virus particle and although a virus particle with only one intact hemolysin unit can lyse rbc, it does so at a much slower rate than a virus with 15 intact hemolysin units.

The results of neutralization of the NDV hemagglutinin and hemolysin by antiserum (Fig. 1) indicate that either process is inactivated by adsorption of one antibody molecule per virus particle. Since the NDV hemagglutinin is presumed to reside in two sites on opposite sides of the virus, it is not surprising that one antibody molecule adsorbed to one virus particle renders it unable to agglutinate rbc. It is, however, surprising that the hemolysin unit is inhibited by only one antibody molecule per virus particle. It is possible that the 15 hemolysin units are clustered together in such a way that the attachment of one antibody molecule to any one hemolysin unit makes it impossible for the other 14 to function. This seems unlikely since it has been estimated that about 10-20% of the total surface area of the virus particle is covered by the hemolsyin units(4). It is also possible that the virus possesses a single attachment site for the rbc which is not associated with the hemolysin units. Thus adsorption of one antibody molecule to this attachment might then inhibit hemolysis by preventing attachment of the virus to the red cell surface. Rubin and Franklin(6) observed that NDV possesses between 2 and 3 such attachment sites for the host cell although only one antibody molecule adsorbed to the virus particle will inhibit the process of infection and plaque formation. It would seem that NDV has several different attachment sites which serve different purposes and act on different cell receptors.

Summary. The NDV hemolysin is shown to be inactivated by a single antibody molecule per virus. Electron irradiation of NDV indicates that rate of hemolysis of rbc depends on number of intact hemolysin units per virus, whereas ability to lyse rbc, given sufficient time, depends on the presence of only one intact hemolysin unit per virus.

I wish to thank Dr. J. F. McCrea for his helpful advice and for the antiserum used in these experiments. The 87909 NDV strain was obtained from Dr. C. R. Woese.

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Enhancement of Tetracycline Serum Levels with Glucosamine Hydrochloride as Adjuvant. Cross-over Studies in Beagle Dogs. (24297)

ARTHUR R. ENGLISH

Bacteriology Laboratory, Pfizer Therapeutic Institute, Maywood, N. J.

In the effort to achieve greater serum concentrations of antibiotics after oral administration, most research has centered on chemical modification of the antibiotic or the use of adjuvants. As an example of the latter, Carlozzi has shown that serum levels of tetracycline* in man were enhanced after oral administration by the use of the amino sugar, D-glucosamine as an adjuvant(1). Data on serum concentrations after oral administration

* Registered trade mark of Chas. Pfizer and Co., for tetracycline is Tetracyn.

TABLE I. Tetracycline Hydrochloride plus Glucosamine Hydrochloride and Tetracycline Hydrochloride. Comparison of serum concentrations after oral administration in a cross-over experiment in 40 beagle dogs.

Sample time	Biological activity* expressed in μg/ml after oral admin.† of Tetracycline hydrochloride	Tetracycline hydrochloride + glucosamine hydrochloride	% increase	Level of significance, %
1	1.123	1.972	76	1
3	1.114	1.512	36	5
5	.878	1.164	33	5
7	.566	.759	34	5

* Avg of 40 dogs. † 10 mg/kg.

of tetracycline hydrochloride plus glucosamine hydrochloride† as compared to tetracycline hydrochloride itself in a cross-over experiment in 40 beagle dogs will be presented here.

Methods. The beagles used in this study were pure-bred and were maintained under identical housing conditions, diet, and environment. Tetracycline hydrochloride plus D-glucosamine hydrochloride in a 1:1 ratio (10 mg plus 10 mg) was used as the test drug. Tetracycline hydrochloride of the same lot was used as the control drug. Dosage was 10 mg/kg administered by stomach tube. Blood samples were removed by jugular vein puncture at 0, 1, 3, 5, and 7 hours after drug administration. Immediately after drawing, the blood was permitted to clot at refrigerator temperature. The serum was then removed and assayed by standard microbiological procedures(2). Such biological activity in the serum has been expressed as μg/ml of tetracycline hydrochloride. The study was done with groups of 20 animals per experiment, that is, 10 on test and 10 on control drug. After a weeks' rest, the cross-over was initiated in which the previous test animals now served as controls and *vice versa*. A zero time sample was always assayed to exclude any possibility of carry-over from the previous week's experiment. The data were subjected to standard analysis of variance for a cross-over design experiment(3) and per cent increase in serum levels noted at one and 5% levels of significance.

† Trade mark of Chas. Pfizer and Co., for dosage form of tetracycline hydrochloride and glucosamine hydrochloride is Cosa-Tetracyn.

Results. The data have been summarized in Table I. Higher biological activity in the sera was observed at every time interval in the dogs receiving tetracycline hydrochloride plus glucosamine hydrochloride than in those animals receiving only tetracycline hydrochloride. Percentage increases in serum activity ranged from 76% at one hour, to approximately 34% at 3, 5, and 7 hours. An analysis of variance for such a cross-over experiment showed the levels of significance for these increases were one per cent at one hour, and 5% at 3, 5, and 7 hours. Although the exact mechanism for enhancement of serum levels after oral administration of tetracycline hydrochloride by D-glucosamine hydrochloride remains unclear, it is obvious that the greatest effect occurs in the early hours after administration.

Summary. Serum levels of tetracycline activity were enhanced by the presence of D-glucosamine hydrochloride from 76% at one hour to 34% at 7 hours over those obtained after oral administration of tetracycline hydrochloride alone in a cross-over experiment with 40 pure-bred beagle dogs.

I am indebted to Miss Dorothy Hucke, Biochemical Research and Development Division, Chas. Pfizer and Co., Inc., for statistical analyses of these data. The author also wishes to express his gratitude for technical assistance of: Misses Sophia Szendy, Diane Shorenstein and Judith Morse, and the Mesdames Rochelle Warhaftig, Mary Jane MacGregor, and Doris Uhoda.

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Distribution and Valence State of Radiochromium in Intracellularly Labeled Ehrlich Mouse Ascites Carcinoma Cells.* (24298)

P. C. RAJAM AND ANNE-LOUISE JACKSON (Introduced by W. S. Preston)
Dept. of Bacteriology, University of Michigan Medical School, Ann Arbor

Gray and Sterling(1) reported a method for intracellular labeling of human erythrocytes with radioactive sodium chromate ($Na_2Cr^{51}O_4$). This method has been further studied and applied by several workers(2,3), resulting in adoption of the modified procedure as a standard method for study of erythrocyte life span and related investigations(4). Any procedure resulting in an intracellular label, stable for the life of the cell, has potentialities for the study of cellular biology. Thus, in addition to those used for erythrocytes, similar methods have been applied successfully to leucocytes(5) and platelets(6). Recently, the use of radiochromate for labeling cells of Ehrlich mouse ascites carcinoma has been reported(7). It was shown that the label was retained intracellularly until irreversible rupture of the cell occurred, and that incorporation of chromate ions in the cells did not impair their ability to produce subcutaneous tumors in mice, provided a certain maximum concentration of chromate ion was not exceeded. In their original paper, Gray and Sterling(1) studied the nature of intracellular label in erythrocytes, and demonstrated that the erythrocyte membrane allowed passage of the CrO_4'' ion, but was selectively impermeable to trivalent chromic (Cr^{+++}) ion. Radioactivity within the red cell was shown to be associated with the globin moiety of the hemoglobin molecule. It was also suggested, on the basis of indirect evidence, that the CrO_4'' ion was intracellularly reduced to the trivalent Cr^{+++} ion, and that the stability of the label was dependent on the firm binding of the Cr^{+++} ion to intracellular protein. Tumor cells are considerably more complex entities than the highly specialized erythrocyte. A study of the valence state and intracellular distribution of radiochromium in these cells was considered of interest, and is the subject of this report.

Methods. Propagation, harvesting and standardization of tumor cells. Ehrlich ascites tumor cells were propagated, harvested, freed of erythrocytes and standard suspensions prepared, by methods described elsewhere(8). *Labeling of tumor cells with radiochromate.* A suspension of 5×10^8 tumor cells in 10 ml 0.85% NaCl was labeled as previously described(7), using 0.9 μc of $Na_2Cr^{51}O_4$ (specific activity 39.38 mc/mg, Na_2CrO_4 concentration 0.02 mg/ml; obtained from Abbott Laboratories under the trade name Rachromate). Unincorporated radiochromate was removed by washing thrice with 0.85% NaCl (35 G, 0°C, 5 min). *Fractionation of tumor cells.* The labeled tumor cell suspension was fractionated by a method based on that of Hogeboom and Schneider(9). Cells were suspended in 0.25 M sucrose solution and disrupted by grinding in a Potter-Elvehjem homogenizer (60 min, 0°C). An aliquot of the total homogenate was removed for radioassay. The remainder was layered on 0.34 M sucrose solution and centrifuged (450 G, 0°C, 15 min). The supernate (SI) was reserved, the sediment suspended in 0.25 M sucrose solution, layered on 0.34 M sucrose solution and centrifuged (800 G, 0°C, 15 min). The sediment, consisting largely of nuclei, was dispersed in sodium desoxycholate solution, and aliquots prepared for radiassay; the supernate (SII) was combined with the original supernate (SI). The combined supernates,

* This work was supported by grants from Mich. Memorial Phoenix Project.

representing the cytoplasmic fraction, were centrifuged (13,000 G 0°C, 30 min). The sediment, consisting of mitochondria and microsomes, was dispersed as above, and aliquots prepared for radioassay. The clear supernate (SIII), containing soluble protein and other metabolites, was divided into 4 portions. The first portion was treated with trichloroacetic acid (final concentration 5%), and the second with absolute ethanol (final concentration 50%) for 1 hour at 37°C. The precipitated protein in each case was separated by centrifugation (900 G, 0°C, 15 min) and dissolved in 10 M urea solution; aliquots of these solutions, and of the respective supernates, were prepared for radioassay. The third portion of the supernate (SIII) was deproteinized with ethanol as described above and the protein-free supernate dialyzed exhaustively against distilled water (30 bag revolutions/minute, 24 hours). Aliquots of the dialyzed material were used for radioassay. *Determination of valence state of intracellular chromium.* The final portion of the supernate (SIII) was used for this purpose. The method was based on that used by Cunningham, McGuir, and Clement(10). Following ethanol precipitation, one ml of a 5% solution of carrier (nonradioactive) chromic chloride ($CrCl_3$) was added and, after equilibration of radioactive and carrier Cr^{+++} ions, both were precipitated with ammonium hydroxide. The precipitate was washed with distilled water (100 G, 0°C, 5 min) and redissolved in 1N HCl. Aliquots of the solution of the precipitate, and also of the combined supernate and washings, were used for radioassay. *Method of radioassay.* The number of counts/minute for all samples were determined in a well-type scintillation counter (Nuclear Model DS-3).

Results. The following results are based on average figures from 3 experiments. The total homogenate obtained from 5 x 10^8 tumor cells labeled with 0.9 μc of $Na_2Cr^{51}O_4$ (approximately 88,000 counts/minute) assayed 18477 counts/minute. This corresponds to approximately 21% uptake, a value which is in accord with previously published results(7). Fractionation of the homogenate into nuclear and mitochondrial-microsomal fractions showed that 17 and 12% respectively of the radioactivity of total homogenate were associated with these fractions. Thus, 71% of the total intracellular activity was present in the soluble cytoplasmic fraction.

TABLE I. Intracellular Distribution of Radiochromium in Ehrlich Mouse Ascites Tumor Cells.

Fraction	Radiochromium Counts/min.*	% uptake*	Avg % uptake
Total homogenate	20,517	100	100
	20,344	100	
	18,402	100	
Nuclear fraction	3,936	19	17
	3,560	17	
	2,984	16	
Mitochondrial-microsomal fraction	2,673	13	12
	2,530	12	
	2,205	12	
Soluble protein†	7,775	38	36
	7,055	35	
	6,582	36	
Protein-free supernate†	5,961	29	29
	6,251	31	
	6,405	28	

* Results of individual experiments.
† Ethanol precipitation.

Trichloroacetic acid precipitation of supernate SIII indicated that 2450 counts/minute (13%) were present in the protein fraction, and 8822 counts/minute (45%) in the protein-free supernate. When ethanol was added as the precipitating agent, 36% of radioactivity was associated with the protein fraction, and 29% with the protein-free supernate. Percentage figures so far presented refer to per cent radioactivity of total homogenate. Expressed in terms of total radioactivity of supernate SIII, ethanol precipitated 52% with protein, while 46% remained in the supernate. Results of individual experiments are summarized in Table I.

Dialysis of the protein-free supernate (ethanol precipitation) showed that, of 6205 counts/minute before dialysis, only 70 counts/minute remained after dialysis (1% of radioactivity of undialyzed protein-free supernate).

Results of the determination of the amount of intracellular chromium present as Cr^{+++} ion are presented in Table II. Practically all the radioactivity of the protein-free supernate was precipitable as Cr^{+++} ion.

Discussion. An examination of the results

TABLE II. Proportion of Radioactivity of Protein-Free Supernate Due to Cr^{51+++} Ion.

Fraction	Counts/min.*	% of total*	Avg % of total
Protein-free supernate	7,426	100	100
	8,048	100	
	6,488	100	
Chromic hydroxide precipitate	6,929	93	94
	7,702	95	
	6,188	95	
Supernate and washings	434	6	5
	292	4	
	257	4	

* Results of individual experiments.

indicated that, in accordance with results previously obtained, tumor cells allow passage of CrO_4'' ions into the cell. Once within the cell, however, hexavalent chromium (CrO_4'') was apparently reduced to the trivalent chromic form (Cr^{+++}). Since the procedure used for precipitation of Cr^{+++} ions from the protein-free supernate involved the possibility of solution of a small portion of the precipitated chromic hydroxide in the first excess of ammonium hydroxide, it is probable that the small amount of total radioactivity not precipitable as Cr^{+++} may be accounted for in this manner. Since these results are in accord with observations on the nature of intracellular label in erythrocytes, and since the tumor cell is permeable to CrO_4'' ions, it appears unlikely that both valence states of chromium coexist in the cell.

The discrepancy between results of trichloroacetic acid and ethanol precipitation of protein was explainable on the basis of the low pH associated with trichloroacetic acid precipitation, since this may bring about release of portein-bound Cr^{+++} ions into the supernate. Ethanol was therefore considered to be the precipitant of choice, and results obtained by alcohol precipitation considered the more reliable.

Part of the radioactivity of nuclear and mitochondrial-microsomal fractions was undoubtedly due to entrained or adsorbed radioactive cytoplasmic protein, and part to nonspecific adsorption of free Cr^{+++} ions to the protein surface components of the particulates in these fractions. In the case of the nuclear fraction, a portion of the radioactivity was probably concentrated in the small number of unbroken cells which are invariably carried down in this fraction. No attempts were made to investigate the possibility of a specific concentration of radiochromium within nuclei, mitochondria, or microsomes.

A point not previously examined, to our knowledge, is that although a large percentage of intracellular Cr^{+++} ion is associated with soluble cytoplasmic protein, a significant amount may be present as unbound Cr^{+++} ion, at least in Ehrlich tumor cells, thus suggesting that the cell membrane may be impermeable to intracellular chromic ions. This suggests that the stability of the intracellular label may not be entirely dependent on protein binding, but is also influenced by membrane permeability. This conclusion is strengthened by direct evidence showing that Cr^{+++} ions do not penetrate the erythrocyte membrane(1), and that radioactivity is almost entirely retained within Ehrlich tumor cells until they are lysed by sonic or other disruption, when almost all of it is released(7).

Summary. 1. Ehrlich mouse ascites carcinoma cells were labeled with radiochromate ($Na_2Cr^{51}O_4$), and the intracellular distribution and valence state of radiochromium studied. 2. Of the total intracellular radioactivity, 71% was associated with the soluble cytoplasmic fraction; of radioactivity of this fraction, ethanol precipitated 52% with the proteins, and 46% remained in the supernate from ethanol precipitation in dialyzable form. 3. Hexavalent chromium ($Cr^{51}O_4''$) appeared to be intracellularly reduced to the trivalent state (Cr^{51+++}). 4. The results suggest that the cell membrane of the Ehrlich ascites tumor cell is impermeable to intracellular Cr^{+++} ions, and that stability of the intracellular label is not dependent on protein binding alone.

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Impairment of Innate Resistance by Triiodothyronine.* (24299)

WILLIAM H. MURPHY, JR.,† ALVIN L. WIENS AND DENNIS W. WATSON
Department of Bacteriology and Immunology, University of Minnesota, Minneapolis

Lurie and Ninos(1) observed that rabbits treated with triiodothyronine displayed an enhanced resistance to chronic infection by tubercle bacilli. This paper reports the effect of triiodothyronine on experimental infection of mice by a strain of group A streptococci, by *Candida albicans,* and on transplantable mouse leukemia.

Materials and methods. L-3,3′,5 triiodothyronine (Smith, Kline & French Co.) was prepared for inoculation of mice by dissolving 4 mg in 1.3 ml 0.01 N NaOH, 90 mg of NaCl added, and total volume of solution brought to 10 ml with distilled water free of carbonate. Triiodothyronine solution (T3) after sterilization by autoclaving for 30 minutes at 110°C., was stable for 6 days at 4°C. *Streptococcus pyogenes,* type 18, and *Candida albicans,* strain 780, were grown and prepared for inoculation of mice as described elsewhere (2,3). Swiss albino mice, Tumblebrook strain, and BALB/C mice, purchased from Jackson Memorial Laboratory, were used as test hosts for *Candida albicans* and *Streptococcus pyogenes* respectively. *Strain C58 mice and transplantable leukemia line I_b* originated with MacDowell and have been maintained in our laboratory since 1952. Young C58 mice moribund from growth of I_b cells served as donors of malignant leukocytes used for tests. Spleens harvested aseptically from leukemic mice were transferred to a sterile Petri dish and minced with Bard-Parker blades until particles were approximately 2 mm^3. Minced tissue flooded with Scherer's (4) maintenance solution (MS) was agitated gently and the supernatant fluid discarded. Tissue was minced further until particles approximated 1 mm^3, supernatant fluid transferred aseptically to a sterile tube, centrifuged to sediment gross particles, and supernate saved as the source of leukemic cells. White blood cells were separated from erythrocytes by cyclic differential centrifugation at low speed(5); line I_b leukocytes comprising the sedimented fraction were suspended in MS and counted in a hemocytometer. Per cent viability of cell suspensions was determined by incubation of an aliquot of cells with an equal volume of 0.2% trypan blue (dissolved in distilled water containing NaCl 0.8%, KCL 0.04%, glucose 0.1%); unstained cells were considered viable(6). Cell suspensions prepared as described above did not contain more than 5% dead cells.

Results. Accelerative effect of triiodothyronine on streptococcal infection of mice. The extraordinary sensitivity of male BALB/C mice to infection by *Streptococcus pyogenes,* type 18, provided an ideal experimental system to assess the effect of T3 on infection. Triiodothyronine, 0.1 ml (40 μg) was inoculated subcutaneously (SQ) into each of 6 test mice 24 hours before they were infected by intraperitoneal inoculation with streptococci as described previously(2). After inoculation, mice received 0.1 ml injections of T3 at 48 hour intervals during the 6-day observation period; control mice were inocu-

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† Present address: Dept of Bacteriology, Univ. of Michigan, Ann Arbor.

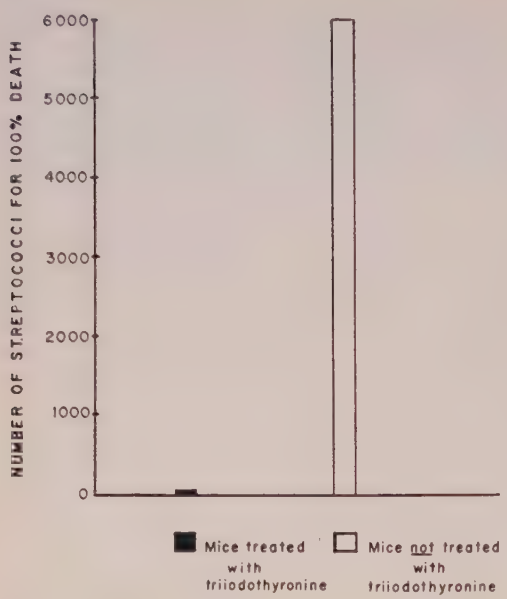

FIG. 1. Effect of triiodothyronine (40 μg) given SQ every 48 hr on time for death of male BALB/C mice infected intraper. by *Streptococcus pyogenes*, type 18.

lated only with streptococci since tests revealed that T3 was not lethal for mice nor did its vehicle influence host response to infection. Representative results from 3 replicate experiments (Fig. 1) show that mice treated with T3 died within 24 hours after inoculation with 6 to 10 organisms whereas approximately 6000 organisms were required to kill untreated mice under the same experimental conditions. Because male BALB/C mice were uniformly susceptible to lethal infection by the test organism, the total number of deaths in test and control groups was the same at the end of the 6 day observation period. *Effect of triiodothyronine on susceptibility of Swiss and BALB/C mice to infection by Candida albicans.* The high innate resistance of mice to infection by *Candida albicans* contrasted sharply with the extraordinary susceptibility of BALB/C mice to test streptococci. Thus, further characterization of the influence of T3 on innate resistance was accomplished by assaying its effect on the course of experimental infection of Swiss albino and inbred BALB/C mice by *Candida albicans*. Procedure for growth of organisms and inoculation of mice has been reported(3). Twenty mice in each test group were inoculated with 0.5 ml of cell suspension containing 35 million organisms. Test mice received 40 μg of T3 subcutaneously 24 hours before inoculation with cells, and every 48 hours thereafter; control mice were inoculated with *Candida albicans* alone. Experiments with each strain of mice were conducted in duplicate. Data (Fig. 2) representative for experiments that employed both BALB/C and Swiss mice reveal that T3 increased per cent mortality and accelerated rate of death. *Effect of triiodothyronine on rate of death from transplanted leukemia.* The possible effect of T3 on innate resistance to neoplastic growth was surveyed by study of its influence on progression of spontaneous leukemia in C58 mice. Results of such experiments were subject to broad variation because it was impossible to allocate control and test mice into equivalent groups comprised of animals at the same stage of leukemia. Analysis of the dose-response curve of C58 mice to line I_b cells (7) afforded an opportunity to assess influence of T3 on host response to transplanted leukemic cells. Three groups of 10 test and 10 control animals per group were employed for comparative purposes; mice of each group received a graded dose of I_b cells. Forty μg

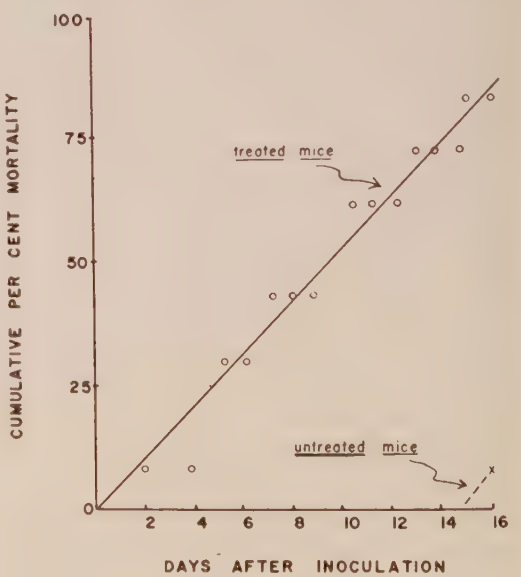

FIG. 2. Effect of triiodothyronine (40 μg) given SQ every 48 hr on time for death of BALB/C mice infected intraper. by *Candida albicans*,

TABLE I. Effect of Triiodothyronine on Time for Death of C58 Mice to Transplantable Leukemia, Line I_b.

Dose of cells per mouse	Avg time in days for death*	
	Treated mice	Control mice
$10^{4.7}$	6.0	7.0
$10^{3.3}$	7.0	9.0
$10^{2.7}$†	12.0	13.3

* Data are representative of 3 replicate experiments; 10 mice were used in each test and control group for each exp.

† Time for death of animals (*ca.* 50% of test subjects) that received leukemic cells.

of T3 was inoculated SQ into mice 24 hours before intraperitoneal inoculation of I_b cells and every 48 hours thereafter; control mice did not receive T3. In 3 replicate experiments a small but consistent accelerative effect of T3 on time for death from transplanted leukemic cells was observed (Table I) although the magnitude of the change was not remarkable.

Discussion. The ready suppression of innate immunity by a variety of experimental procedures contrasts with the lack of suitable methods for enhancement of native resistance. Lurie and Ninos(1) demonstrated that triiodothyronine augmented innate resistance of rabbits to chronic tuberculosis. Long and Sewell(8) observed that injection of thyroxine into guinea pigs increased immunity to diphtheria toxin and resulted in increased production of circulating antitoxin. These findings served as the basis for the study reported here which had as its objective a further survey of the effect of triiodothyronine (T3) on innate immunity. Contrary to anticipated results, it was found that mice treated with T3 were more susceptible to infection by a strain of group A streptococci and by *Candida albicans* than untreated controls, whereas time for death of mice from transplanted leukemia was only slightly accelerated. In experimental candidiasis both threshold of resistance and time for death were reduced. These observations are consistent with those of Nutter and coworkers(9) and Melby, *et al.*(10), who reported suppression by triiodothyronine of innate resistance to infectious agents. Three factors seem to be important to an understanding of the divergent results concerning the effect of T3 on innate immunity: 1) the test systems employed in the various investigations serve to illustrate the individuality of each host-parasite system; 2) the dose of test organisms influenced the magnitude of the observed effect in at least one case(10), and 3) the dose of T3 may augment resistance providing that it enhances the physiologic and metabolic activity of cells concerned in resistance to infection, or it may subvert innate immunity if used in doses that are thyrotoxic.

Summary. Studies were carried out to provide additional information on the effect of triiodothyronine on innate resistance. Mice treated with the drug were significantly more susceptible to infection by *Candida albicans* and by a strain of *Streptococcus pyogenes,* type 18, than untreated controls. Treatment of mice with triiodothyronine failed to enhance significantly time for death of C58 mice to transplanted leukemia, line I_b.

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Formation of Protoplasts from Mycobacteria by Mycobacteriophage.*† (24300)

IRVING MILLMAN‡
Department of Microbiology, Northwestern University Medical School, Chicago

Isolation of both enzymatically active and immunogenically active particles from tubercle bacilli has been reported(1,2,3,4). Isolation of these intracellular units, resembling mitochondria in many respects, was obtained by grinding whole washed cells with abrasives in a ball mill for as long as 18 hours. This procedure was drastic but necessary since other means resulted in failure(5). Attempts to correlate particulate fractions with some intracellular structure(2,3) have met with criticism since, as has often been pointed out, cell walls as well as cell membranes could be ground to heterogeneous particulates resembling in size and sedimentation rate the particles referred to as mitochondria(6). The solution to this problem with tubercle bacilli would be to use less drastic means for liberation of intracellular components. The conversion of tubercle bacilli to protoplasts is the obvious answer but unfortunately this group of microorganisms is resistant to the lytic action of lysozyme(7) and therefore, cannot be converted by this means.

This report will describe a method successfully used in this laboratory for conversion of different strains of tubercle bacilli to protoplasts by use of concentrated mycobacteriophage.

Materials and methods. For stock suspensions of phage§ trypticase soy agar plates were seeded with sufficient numbers of the avirulent *Mycobacterium tuberculosis* strain 607 and phage to produce confluent lysis in 24 hours at 37°C. The surface from 25 such plates was washed with 0.85% NaCl and the pooled washings filtered through Berkefeld filters.

An adaptation procedure was necessary in order to produce phage active in forming protoplasts of the different strains tested. It was found that phage propagated on strain 607 had little or no activity against the H37Ra, BCG‖, and H37Rv mycobacterial strains while phage propagated on strain H37Ra was active against strains BCG and H37Rv as well. The following procedure was followed. Two 4 liter Erlenmeyer flasks each containing 2 liters of Proskauer and Beck medium were inoculated with 0.075 mg of strain 607 or H37Ra (determined by centrifugation in a Hopkins tube) and 1 ml of stock 607 phage containing 3 x 10^4 phage. This represented a phage bacteria ratio of approximately 1-100. The flasks were then stoppered with rubber stoppers containing cotton adapters. These were placed on a shaking machine housed in a 37°C incubator and the flasks were agitated gently for 48 hours when strain 607 was used and for 3 weeks when strain H37Ra was used. Periodically aliquots from the strain 607 flasks were removed and titered by plaque count against the 607 strain. The H37Ra strain was titered in the same manner only once at the end of the 3 weeks incubation period and only against the H37Ra strain. The culture medium was then filtered through Berkefeld filters, shell frozen in lyophilizing flasks and evaporated under vacuum for 24 hours. This produced a 10-fold concentration (4 liters to 400 ml). The 400 ml were then dialyzed at a temperature of 5°C against 0.01 M phosphate buffer at pH 7.0 (3 changes over a period of 24 hours) and lyophilized to dryness. The dry material was resuspended in a desired volume of 10% sucrose containing 0.1% $MgSO_4$. The number of cells to be converted to protoplasts determined final volume

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‡ Present address: Public Health Research Institute of the City of New York, Inc.

§ Obtained from Dr. S. Froman, Dept. of Bacteriology, Univ. of California, Los Angeles.

‖ Obtained from Dr. S. Rosenthal, Tice Clinic, Univ. of Illinois, Chicago.

of phage concentrate. As a rule 10 ml was the final volume chosen. These produced an estimated phage concentrate of 10^8. This count was estimated entirely on the basis of volume since it was found that the treatment necessary for concentration inactivated approximately 90% of the original phage particles. This was evidenced by a reduction in the formation of plaques after incubation with either the 607 or H37Ra strain of tubercle bacilli as host. Four ml of the phage concentrate was added to 0.01 mg wet weight of bacterial cells suspended in 6.0 ml of 10% sucrose containing 0.1% $MgSO_4$. This allowed a phage bacteria ratio of 1000:1. The mixed suspension was incubated in a water bath at 37°C and examined in wet mount continuously for 5 minutes and every 15 minutes thereafter for a total of 60 minutes.

Light micrographs were taken with a Spencer dark M objective (N.A. 1.25) at 1/100 sec. using Adox KB 14 35 mm film. For electron micrographs a microdrop of suspension obtained at specified times was placed on a formvar grid and allowed to air dry. This was then washed by adding and withdrawing a drop of distilled water from a dropper 10 to 20 times. The prepared grids were then examined in the EMU-3C electron microscope.

Results. Fig. 1-7 represent photomicrographs of the 607 strain of tubercle bacilli. After 15 minutes of contact with phage concentrate at 37°C changes in mycobacterial cell morphology occurred (Fig. 1, 2, 5). At the start of observation the cells appeared more refractive than normal cells (Fig. 1) with a surrounding halo of brilliant light. Although swelling was evident after only 5 minutes it was very pronounced after only 15 minutes. Swelling began either at the ends or in the center of individual cells. Often, as a result of swelling at one end only, the cell would appear to be bent at right angles or to assume the appearance of a figure eight (Fig. 2). The figure eight type would always remain constricted in the center and eventually form 2 protoplasts (Fig. 3). Where only one end would swell the other end would remain attached (Fig. 3). At the end of 60 minutes complete protoplasting was evident (Fig. 4). The individual protoplasts remained surrounded by a halo of light making it difficult to visualize the true outline of the cell. Fig. 6 shows 2 protoplasts surrounded by debris and/or phage particles. The photograph appears blurred due to the presence of sucrose which was difficult to remove completely without destroying the labile unfixed protoplasts. While the technic used did not allow detection of phage particles attached to cell walls it is felt that newly reported fixation procedures(8) will permit this to be accomplished.

Similar results were obtained with the H37Ra, H37Rv and BCG mycobacterial strains.

Discussion. The results reported confirm those of Carey *et al.*(9) of formation of *Escherichia coli* protoplasts by the mass absorption of inactive phage. Although the preparation of their phage concentrate differed from that reported here the results are essentially the same.

Of interest is the fact that the phage, which was non-specific in its ability to lyse different strains of tubercle bacilli, as shown by formation of plaques on Petri plates, should require a period of adaptation to be capable of causing formation of protoplasts of the H37Ra, H37Rv and BCG strains of tubercle bacilli. The reason for this is still obscure but may reflect differences in cell wall composition between the two groups of mycobacteria or differences in phage particles. If the latter is correct the adaptation period was a period of selection where only those phage particles active against strains H37Ra, H37Rv and BCG were selected.

While it appears likely that the protoplasts were caused by mass adsorption of inactive mycobacteriophage causing a weakening and dissolution of cell walls it also remains a possibility that some component(s) liberated from the phage by the concentration treatment was responsible. Whatever the mechanism, the fact remains that the ability to convert mycobacterial cells to protoplasts will simplify future studies on relation of intracellular structure and function among the

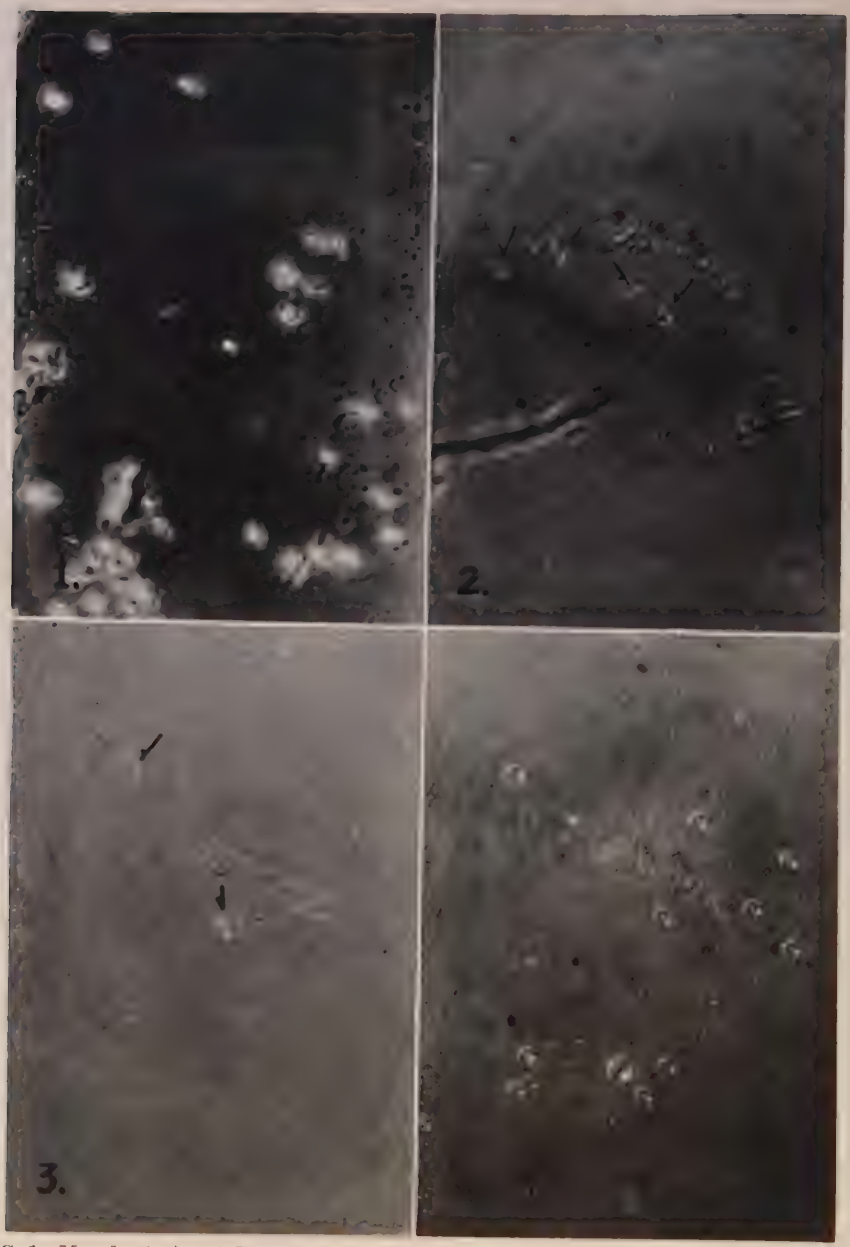

FIG. 1. *Mycobacterium tuberculosis* strain 607 after 15 min. contact with phage concentrate. Individual cells highly refractive and swelling evident either at one end or both ends of cell. A normal cell (*n*) visible for comparison. × 3300.

FIG. 2. *M. tuberculosis* strain 607 after 15 min. contact with phage concentrate. Figure eight and bent forms visible. × 3300.

FIG. 3. Same after 30 min.

FIG. 4. Same after 60 min.

mycobacteria.

Summary. Various mycobacterial cells were converted to protoplasts by treatment with a mycobacteriophage concentrate. The technic involved production of sufficient adapted phage by growth either in the 607 or H37Ra strains of tubercle bacilli. Further concentration was necessary in order to obtain a

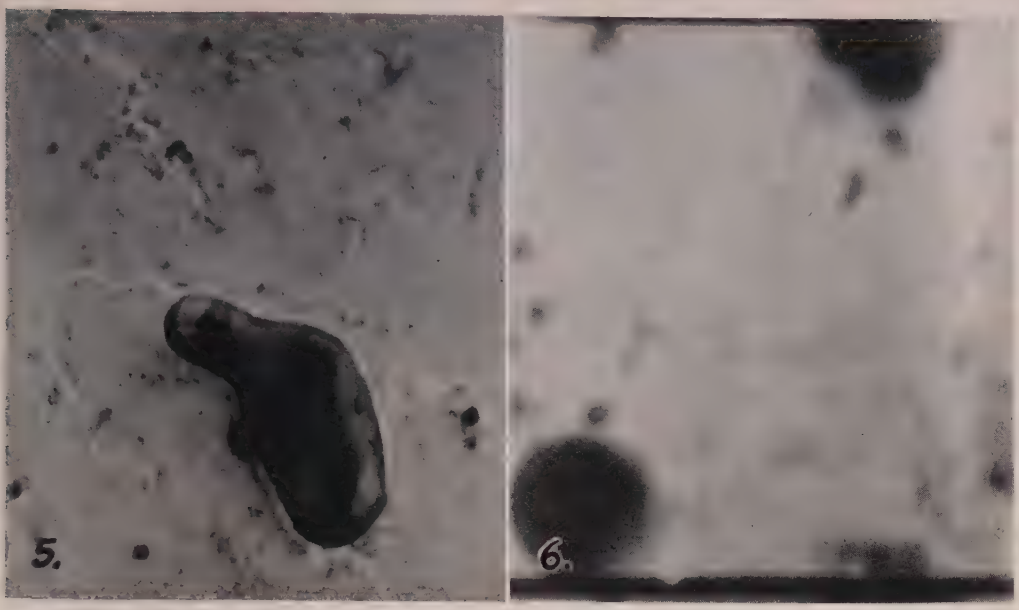

FIG. 5. Swollen cell of *M. tuberculosis* strain 607 after 15 min. contact with phage concentrate. × 36514.

FIG. 6. Protoplasts of *M. tuberculosis* strain 607 after 60 min. contact with phage concentrate. × 25025.

sufficiently high phage bacteria ratio. This was accomplished by lyophilization. The significance of the reported findings is discussed.

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o-Hydroxyphenylacetic Acid Excretion in the Phenylalanine Tolerance Test For Carriers of Phenylketonuria.* (24301)

Andrea M. Cullen and W. Eugene Knox (Introduced by Shields Warren)

Cancer Research Institute, New England Deaconess Hospital and Dept. of Biology and Chemistry, Harvard Medical School, Boston, Mass.

The recessive gene responsible for phenylketonuria is associated in the heterozygous parents with an abnormally low tolerance for ingested phenylalanine(1) and an elevated fasting level of plasma phenylalanine(2). This accumulation of phenylalanine in the apparently normal carriers of the disease is referable to a partial impairment of phenylalanine hydroxylase, the enzyme whose ab-

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sence from the liver of the homozygous phenylketonuric individuals(3,4,5) may account for all of the biochemical and clinical abnormalities of the disease(6). The reduced phenylalanine tolerance of carriers of the phenylketonuric gene was confirmed by Berry, Sutherland and Guest(7), who also reported that o-hydroxyphenylacetic acid (o-HPA) was excreted in the urine of the heterozygotes, but not of normals, after phenylalanine was administered in test doses. No other metabolites characteristic of phenylketonuria were found regularly in these experiments. The suggestion was made that the presence of o-HPA in urine during tolerance tests could perhaps serve as an added means of identifying the carriers of the recessive gene. o-HPA is the major abnormal hydroxyphenyl metabolite excreted in phenylketonuria(8) and its increased formation has been attributed to the action of the same genetically controlled enzyme whose normal *para*-hydroxylation of phenylalanine is impaired(9). If this were so, its appearance in the urine of heterozygotes would represent a qualitative test for the presence of the genetically distorted enzyme reaction, a test superior to the quantitative tests now used. It would also indicate the kind of chemical alteration which affects the enzyme in this disease. On the other hand, o-HPA formation may occur by an independent system in proportion to the height of the blood phenylalanine level. In phenylketonuria the excretion of o-HPA is proportional to the blood phenylalanine level(8). The following experiments demonstrate that the same proportionality of o-HPA excretion to blood phenylalanine level holds in normal individuals, and that o-HPA formation is not qualitatively dependent upon the presence of the phenylketonuric gene.

Methods. Four young adult members of the laboratory staff, 3 males and 1 female, were subjects. All were non-heterozygotes as judged by normal basal levels of plasma phenylalanine, and 2 of these, previously tested, showed normal phenylalanine tolerance curves. The curve on one of these (PI) was at the high extreme of the normal range. In the present study these subjects were given by mouth an amount of phenylalanine calculated to raise the phenylalanine in their blood to a level comparable to that which the heterozygote would reach with 0.1 g/kg. The subjects were not fasting. After ingestion of the designated amount of L-phenylalanine, blood samples and urine specimens were obtained at hourly intervals for 3 hours. Phenylalanine in plasma was determined by a modification(2) of the decarboxylase method of Udenfriend & Cooper(10). o-HPA was measured by paper chromatography of ether extracts of the urine, following the method described by Berry, Sutherland and Guest (7). The only modification of this technic consisted in using twice the amount of concentrated urine extract (equivalent to 400 μg of creatinine) for each spot on the chromatograms.

Results. We have confirmed the findings of Berry, Sutherland and Guest(7) on 14 heterozygotes and 9 controls that after 0.1 g/kg of L-phenylalanine usually only the heterozygotes excrete detectable amounts of o-HPA. The blood levels of phenylalanine expected after this dose of phenylalanine in the two types of individuals are shown by the dotted lines in Fig. 1, representing the

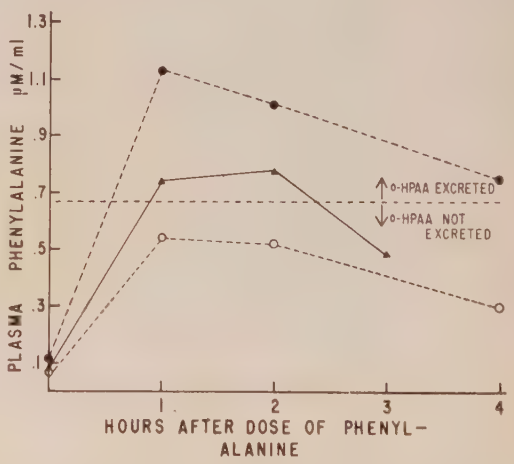

FIG. 1. Plasma phenylalanine following oral doses of L-phenylalanine in 19 controls (–○–) and 19 heterozygotes (–●–) given 0.1 g L-phenylalanine per kg of body wt, and in 4 normal subjects (–▲–) given 0.1 to 0.13 g L-phenylalanine per kg body wt (Table I). Dotted line at 0.67 μm/ml represents "threshold" of plasma phenylalanine above which excretion of o-HPA was observed by the method used.

TABLE I. Plasma Phenylalanine and Urine o-HPA.

Subject	Phenylalanine ingested, g/kg body wt	Plasma phenylalanine, μM/ml (after ingestion of phenylalanine)				o-HPA excreted in 3 hr, μg/mg creatinine
		Fasting	1 hr	2 hr	3 hr	
TA	.13	.072	.804	.858	.549	18
PI	.1	.068	.935	.776	.426	21
BE	.13	.075	.544	.803	.489	3
CU	.1	.080	.679	.495	.400	trace
"	.12		.691	.672	.459	4

average curves for 19 individuals in each group(1). Superimposed on this graph is the average of the tolerance curves of the 4 normal subjects after receiving the doses of L-phenylalanine listed in Table I. During the 3 hour period following ingestion all subjects excreted 3 to 21 μg of o-HPA per mg of creatinine. The horizontal line at about 0.67 μmoles/ml represents the "threshold" level of plasma phenylalanine. With values above this level the excretion of o-HPA measured by the present method regularly occurred in both normal and heterozygote individuals. The amount of o-HPA excreted was roughly proportional to the area of the curve above this line. A similar division between the plasma levels of those who did and those who did not excrete o-HPA is seen at about 9 mg% in the data of Berry, *et al.* This and the other values determined by their method were about 30% less than by our method. The excretion of about 1 mg o-HPA per day by normal individuals(8) indicates that more sensitive methods than we employed would detect o-HPA in the urine of individuals whose phenylalanine levels were below the "threshold" line.

In Table I the individual phenylalanine levels of the 4 subjects are given with the sum of o-HPA excreted by each during the 3 hour test period. It is to be noted that normal subject, PI, whose tolerance curve was at the upper range of normal despite a normal basal level, excreted o-HPA after the standard dose of phenylalanine of 0.1 g/kg body weight. Subject CU excreted significant amounts of o-HPA only with a dose of 0.12 g/kg and not after 0.1 g/kg. Even in the latter case traces of o-HPA were found which would not have been detectable with the smaller aliquots chromatographed by Berry, Sutherland and Guest(7).

Discussion. While the heterozygote and the homozygous phenylketonuric have a deficiency in an enzyme system responsible for the metabolism of phenylalanine, they are not unique in disposing of an excess of phenylalanine in part by o-hydroxylation. That this also occurs in the normal is shown in the present work. Under conditions which impose a comparable load of phenylalanine in the blood of the non-heterozygote, the latter also has the capacity to form o-HPA. Isotope studies in the phenylketonuric patient showed that o-HPA was derived from phenylalanine(11). The results of the present study indicate that o-HPA is also derived from phenylalanine in normals and carriers of the phenylketonuric gene. This must occur by another reaction(12), and not by distortion of the phenylalanine hydroxylase reaction. In the presence of elevated blood levels of phenylalanine this second reaction functions as one of the overflow mechanisms to dispose of the excess amino acid. The excretion of o-HPA during a phenylalanine tolerance test reflects an elevated blood level of phenylalanine, and while it is an additional indication of a low tolerance, it is not independent evidence of heterozygosity for phenylketonuria.

Summary. o-Hydroxyphenylacetic acid is an abnormal urinary metabolite of phenylalanine found in phenylketonuria and after phenylalanine dosage in heterozygotes of phenylketonuria. It is also formed by normal individuals with comparably elevated phenylalanine blood levels. It must be formed by enzyme systems other than the one disturbed in phenylketonuria.

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Studies in Leukemia: XI. Active Immunization of C3H x 101 Mice Against Induction of Leukemia.* (24302)

BILL P. MADUROS, STEVEN O. SCHWARTZ AND HAROLD M. SCHOOLMAN
Dept. of Hematology, Hektoen Institute for Medical Research, Cook Co. Hospital, Chicago, Ill.

Our previous reports have described induction of leukemia in C3H mice by means of cell-free brain filtrates(1). Experiments were devised to study the possible immunologic properties of cell-free brain filtrates exposed to ultraviolet light. The present report gives the results of experiments regarding active immunization of mice against leukemia.

Materials and methods. C3H x 101 mice, from 8 to 10 weeks old, served in the experiments. The mice were obtained from Cumberland View Farms, Clinton, Tenn. Cell-free brain filtrates (CFBF) were prepared by method previously described(2). Tumor-cell suspensions were prepared by grinding mesenteric tumor in ground glass tissue mill and suspending the cells in a 0.9% solution of sodium chloride, buffered at pH 7.4, to a concentration of about 10^6 cells/cm^3. Brains of 5 leukemic C3H x 101 mice were pooled; 1000 cc. of CFBF was prepared. A control filtrate was similarly prepared with brains of non-leukemic C3H x 101 mice. The filtrates were stored for 48 hours at –15°C, then thawed 24 hours at 4°C. After filtrates had thawed, the non-leukemic CFBF was filtered through a coarse and a fine glass-sintered filter; the leukemic CFBF was filtered through the same filters. Samples of leukemic CFBF taken before and after this filtration retained their leukemogenic properties when later tested. The filtrates were irradiated with ultraviolet light (UVI) in a Spinco centrifilmer irradiation machine at 1750 rpm at full power and rate of flow of 200 ml/minute.† When irradiation was accomplished, the filtrates were stored in 5 ml and 10 ml vials at –60°C until used. All experimental mice were immunized with ultraviolet irradiated cell-free brain filtrate (UVI-CFBF) by subcutaneous inoculations of 0.1, 0.25, and 0.5 cc at 48-hour intervals. The mice were challenged 10 days after last inoculation, by intraperitoneal (IP) injection of 0.5 cc CFBF or tumor-cell suspension prepared from leukemic C3H x 101 mice. The mice were killed when they appeared terminally ill and autopsies performed. In mice described as positive, both gross and microscopic data regarding leukemia were similar to those described elsewhere(3) for the Swiss strain. Ten C3H x 101 mice were

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† Irradiation of filtrates was supervised by Dr. A. M. Wolf and his staff at Michael Reese Research Fn.

immunized with UVI leukemic CFBF and 10 with UVI non-leukemic CFBF. This experiment was repeated with a different source of leukemic CFBF for challenging. In addition, 2 groups of 10 C3H x 101 mice each, were similarly immunized and challenged with tumor-cell suspension.

Results. All animals that evidenced leukemia did so from 2 to 4 weeks after challenging. For our purpose, mice that survived 20 weeks after having been challenged, were considered negative. No negative animals subsequently showed evidence of leukemia.

Table I summarizes the effect of immunization with UVI leukemic CFBF when mice are challenged with leukemic CFBF. The differences between duplicate experiments are not statistically important; therefore, they are grouped together for final analysis. Protection afforded by immunization with UVI leukemic CFBF is highly significant.

The results of active immunization with UVI-CFBF when challenged with tumor cells are summarized in Table II. Protection is not afforded against a high concentration of tumor cells.

Discussion. Under conditions of our experiment, ultraviolet irradiated cell-free brain filtrate (UVI-CFBF) actively immunizes mice against the leukemogenic agent in leukemic CFBF. That this immunity is not a result of a non-specific mouse brain effect is shown by failure of UVI *non-leukemic* CFBF to confer any significant immunity. The incidence of leukemia in this control group is similar to that reported previously in non-immunized C3H mice(1).

Immunity may not be absolute and may represent only a prolonged latent period; however, in all positive animals, leukemia developed within 4 weeks of challenge whereas the remaining animals survived over 20 weeks. This suggests an absolute immunity at time of challenge.

The failure of UVI-CFBF to protect against tumor cells may be the function of several factors: (1) a possible quantitative relationship of antibody level to the concentration of tumor cells; (2) the inaccessibility of the active agent in tumor cells to the antibody; (3) possible so-called autonomy of the established tumor cells.

TABLE I. Effect of Active Immunization on Induction of Leukemia with *Cell-Free Brain Filtrate.*

Immunized with	No. leukemic / No. immunized		Total
UVI non-leukemic CFBF	8/10	9/10	17/20
UVI leukemic "	2/9 *	1/10	3/19*

CFBF = Cell-free brain filtrate
UVI = Ultraviolet irradiated

Corrected CHI square$_{(1)}$ = 14.44***

* One mouse in this group died of trauma during challenging inj. For statistical analysis, this animal was considered positive and the group restored to 10 animals.

TABLE II. Effect of Active Immunization on Induction of Leukemia with Tumor Cell Suspension.

Immunized with	No. leukemic / No. immunized
UVI non-leukemic CFBF	10/10
UVI leukemic "	10/10

CFBF = Cell-free brain filtrate
UVI = Ultraviolet irradiated

Summary. 1. Leukemic and non-leukemic cell-free brain filtrates were irradiated with ultraviolet light. C3H x 101 mice were actively immunized by subcutaneous inoculations. 2. Active immunization with ultraviolet irradiated leukemic cell-free brain filtrate gave significant protection against challenge of leukemic cell-free brain filtrate. 3. Active immunization with ultraviolet irradiated cell-free brain filtrates did not protect against challenge of a high concentration of tumor-cell suspension.

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Effect of Intrinsic Factor on Vit. B_{12} Uptake by Rat Intestine *in vitro*.* (24303)

ELLIOTT W. STRAUSS† AND T. HASTINGS WILSON (Introduced by Ernest Knobil)
Richard C. Curtis Hematology Laboratory, Peter Bent Brigham Hospital and Departments of Medicine and Physiology, Harvard Medical School, Boston, Mass.

Since the original postulation of Castle(1) concerning factors involved in the etiology of pernicious anemia, many clinical studies have been performed to elucidate the mechanism by which the intrinsic factor stimulates intestinal absorption of B_{12}. More recently, the rat has been useful as experimental animal in study of the problem of B_{12} absorption. A number of attempts to simplify the experimental conditions still further by studying segments of intestine *in vitro* have been unsatisfactory(2,3). A recent report indicated a 2-fold stimulation of B_{12} uptake into isolated rat gut by hog intrinsic factor(4).

The present study deals with an application of the everted sac technic(5) to this problem. Rat gastric juice causes a marked stimulation of B_{12} uptake by rat intestine in this preparation.

Methods. Segments of washed intestine were everted, filled with fluid by means of blunt needle attached to syringe, and tied at both ends as previously described(5). The solution on the serosal side (about 1 ml) consisted of Krebs-Henseleit bicarbonate-saline containing 200 mg% glucose. The filled sac was placed in a 50 ml Erlenmeyer flask with 4 ml of bicarbonate-saline containing the following: 1 ml of neutralized gastric juice (pH 7.4), 1 ml containing 0.005 μg Co^{60} labeled B_{12} (1 $\mu c/\mu g$), in 0.15 M NaCl and 2 ml of a solution such that final concentration of ions was that of Krebs-Henseleit bicarbonate-saline and the final concentration of glucose was 200 mg%. The flasks were gassed with 5% CO_2 and 95% O_2 and incubated with shaking for 1 hr at 30 or 37°C. Both incubation temperatures were found satisfactory although somewhat larger B_{12} uptake was observed at 37°C. The sacs were then removed from the flasks and washed carefully with 3 separate 50 ml volumes of saline, taking care to remove any adhering mucus. The sac contents were removed by needle attached to syringe. The tissue was slit open, blotted on filter paper and weighed. Digestion of the tissue was carried out in 2 ml of concentrated H_2SO_4 at 100°C for 30 minutes and the counting performed in a well-type scintillation counter (with a background of about 100 counts/minute).

Results. Table I shows results of experiment in which 2 sacs of everted rat intestine were incubated for 1 hour with B_{12} on the mucosal side, one in the presence of neutralized gastric juice, the other in the presence of boiled gastric juice (10 minutes at 100°C). Gastric juice stimulated B_{12} uptake into the gut wall by a factor of 11-fold compared with the control. Concentration of B_{12} in the intestinal wall (195 cpm/100 mg tissue) was more than twice that in the final mucosal solution (73 cpm/0.1 ml). Despite the fact that both fluid and glucose moved across the full thickness of the tissue, no B_{12} appeared on the serosal side of the intestinal sac. Over 40 such experiments have been performed with similar results, maximum stimulation of B_{12} uptake by rat gastric juice being 25 fold.

The effect of various locations along the small intestine on B_{12} uptake with and without gastric juice was studied in a further series of experiments. Table II shows that the mid-portion of the rat intestine is susceptible to the greatest stimulation of B_{12} uptake in the presence of intrinsic factor.

Experiments were devised to test the possibility that intrinsic factor was adsorbed or bound in some manner to the intestinal epithelium prior to its reaction with Vitamin B_{12}. One such experiment is given in Table III. One sac of rat intestine was incubated with gastric juice for 45 minutes, washed care-

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† Damon Runyon Cancer Research Fellow.

TABLE I. Effect of Intrinsic Factor on B_{12} Uptake by Sacs of Rat Intestine.

	Intrinsic factor (gastric juice) Sac 1	Control (boiled gastric juice) Sac 2
B_{12} in gut wall (cpm/100 mg tissue)	195	17
B_{12} in serosal solution	0	0
Glucose transport (mg/100 mg tissue)	.95	.99
Fluid movement (ml/100 mg tissue)	.09	.15
Tissue wt (mg)	340	406

Initial B_{12} activity on mucosal side = 85 cpm/0.1 ml sol.
Incubation for 1 hr at 37°C.

fully in saline, and reincubated in a B_{12} solution for a further 60 minute period. Very little B_{12} uptake was observed. A second sac was treated similarly except that the second incubation solution contained intrinsic factor as well as B_{12}. A good uptake of B_{12} was obtained in this second segment of intestine. The pair of sacs incubated in saline acted as controls to demonstrate that incubation followed by washing does not alter the behavior of the tissue. A final sac was incubated first in B_{12} and then, following a saline wash, in intrinsic factor. Little uptake was observed.

Discussion. In the experiments with 1 hour incubation times no B_{12} appeared inside the sac (serosal side) although both fluid and glucose moved across the intestinal wall. This behavior is consistent with the time lag between uptake in the epithelium and appearance in the blood and other organs which occurs *in vivo*(6). Apparently an hour or more is required for passage across the epithelium in the rat. Another feature of this study which agrees with *in vivo* observations is the site in the small intestine of B_{12} absorption. In rats given B_{12} by mouth the midportion of the small intestine takes up considerably larger amounts of B_{12} than either high jejunum or low ileum(6,7). The experiment in Table III clearly shows that intrinsic factor was not adsorbed to the intestinal epithelium as one rinse completely removes its activity from the tissue. Only when B_{12} and intrinsic factor are present simultaneously is there uptake of B_{12} by the intestinal wall.

TABLE II. Effect of Intrinsic Factor (Rat Gastric Juice) on B_{12} Uptake by Various Segments along Rat Small Intestine.

Location		Stimulation of B_{12} uptake by intrinsic factor (% above control)*
High jejunum	(Sac 1)	0
Mid "	2	500
Low "	3	550
High ileum	4	1200
Mid "	5	20
Low "	6	0

* At each location an adjacent sac was used as control, 1 ml 0.9% NaCl substituted for 1 ml gastric juice. Incubation 1 hr at 30°C.

Summary. Sacs of everted small intestine of the rat incubated in bicarbonate-saline containing glucose and B_{12} showed only a very small B_{12} uptake in the absence of a source of intrinsic factor. In the presence of neutralized rat gastric juice the stimulation of B_{12} uptake was 10- to 25-fold. No B_{12} appeared on the serosal side of the preparation. Stimulation of B_{12} uptake was most marked in segments of intestine taken from the midportion of the small intestine. Evidence is presented that neither B_{12} nor intrinsic factor when added alone are adsorbed to the intestinal wall but must be present simultaneously to give B_{12} uptake.

TABLE III. Effect of Prior Incubation of Intestine in Intrinsic Factor on Subsequent B_{12} Uptake.

1st incubation (followed by saline wash)	2nd incubation	B_{12} uptake by intestine (epm/100 mg tissue)
I.F.	B_{12}	12
I.F.	B_{12} + I.F.	104
Saline	B_{12}	7
"	B_{12} + I.F.	100
B_{12}	I.F.	8

Sacs prepared from consecutive segments of midportion of single rat intestine. I.F. = 1 ml of neutralized rat gastric juice. 1st incubation 45 min.; 2nd, 60 min. 37°.

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Relationship of Circulating Antigen-Antibody Complexes, Antigen Elimination, and Complement Fixation in Serum Sickness.*† (24304)

WILLIAM O. WEIGLE‡ AND FRANK J. DIXON

Dept. of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, Pa.

The symptoms and lesions of both clinical and experimental serum sickness usually develop during rapid immune elimination of antigen(1-5). In rabbits, this immune elimination of antigen is associated with circulating antigen-antibody complexes(6,7) and with a fall in serum complement(8,9). The present serologic and histologic studies were undertaken to find whether the level and persistence of the complexes, in the circulation, and/or magnitude of the fall in complement could be correlated with extent of the associated morphologic manifestations of serum sickness.

Materials and methods. Bovine serum albumin (BSA), Armour and Co., Lot No. P67908 was trace-labelled with I^{131} (I*) according to the method described by Talmage *et al.*(10). Twenty-six albino rabbits, weighing approximately 3 kg, were injected intravenously with 250 mg of I*BSA/kg and bled (2.5-3.0 ml sera) every 1 to 2 days starting on either the first or third day following the injection. The sera were analyzed for total I*BSA, I*BSA bound to globulin, and complement. Total I*BSA activity was determined by counting the protein-bound I* in aliquots of sera, following trichloroacetic acid precipitation. These counts were converted to % of injected I*BSA remaining in the total blood volume(10). All samples contained sufficient counts to insure a maximum counting error of less than 5%. I*BSA present as a circulating I*BSA-anti BSA complex was determined by the ammonium sulfate technic of Farr(11). Aliquots of sera were diluted with equal volumes of 0.15 M NaCl and the globulin of these mixtures was precipitated at 37°C with equal volumes of saturated ammonium sulfate. The precipitates were washed twice with 50% saturated ammonium sulfate and were counted for I*BSA activity. The counts were converted to % of injected BSA which was bound to the total serum globulin. The hemolytic activity of the sera (CH_{50}units) was determined by the method of Osler *et al.*(12). All animals which showed an immune elimination of the I* antigen before the sixteenth day following injection were sacrificed 1 day after antigen was eliminated. With 2 exceptions, all other animals were sacrificed on the 16th or 17th day. One rabbit which showed no immune elimination of antigen was sacrificed on the 14th day following injection of the antigen. Another rabbit which did not show an immune elimination until the 18th day was sacrificed one day later. Tissues obtained at autopsy from all animals were fixed in formalin, embedded in paraffin, cut at 5 μ and stained with hematoxylin and eosin for microscopic examination. From every animal the tissues taken for section in-

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‡ Public Health Research Fellow of Nat. Inst. of Neurol. Dis. and Blindness.

cluded one or more blocks from each kidney and at least 2 blocks transecting the base of the heart, including major coronary vessels, cardiac valves, and atrial and ventricular en-

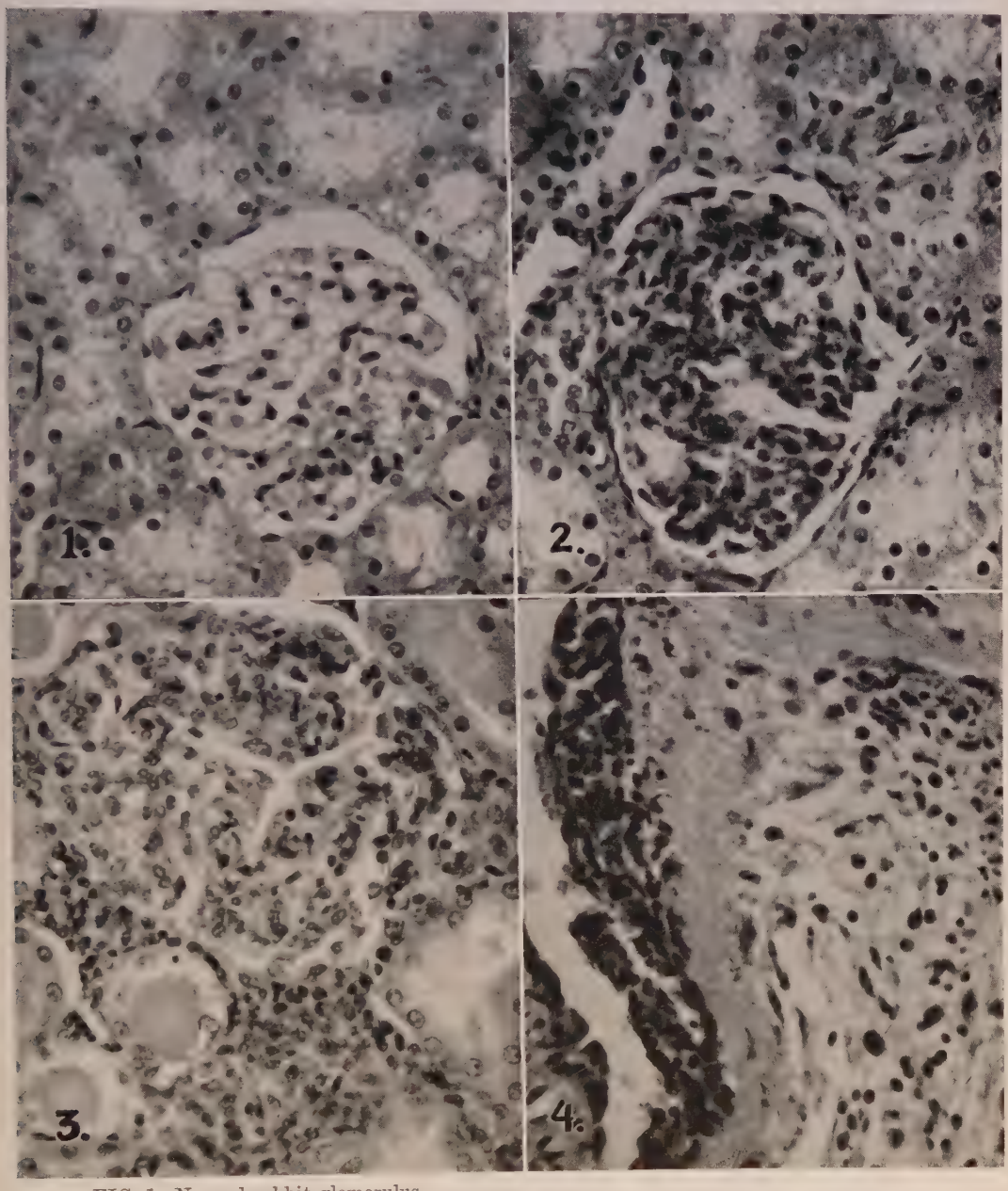

FIG. 1. Normal rabbit glomerulus.

FIG. 2. Glomerulus from rabbit 5151 which had circulating complexes without accelerated elimination of antigen. This is an example of moderate glomerulitis with an increased number of endothelial and epithelial cell nuclei and scarcity of patent glomerular capillaries. Same magnification as Fig. 1.

FIG. 3. Glomerulus from rabbit 5270 showing 3+ glomerulitis. Marked proliferative changes making the glomerulus virtually bloodless. Same magnification as Fig. 1.

FIG. 4. Base of mitral valve leaflet from rabbit 5151. Note proliferation of endocardium and inflammatory infiltrate in substance of valve.

docardium. Sections of these tissues were studied to determine incidence and degree of glomerulonephritis, arteritis, and endocarditis. In accompanying Table: + glomerulonephritis indicates a mild hypertrophy and hyperplasia of endothelial and epithelial cells; ++ glomerulonephritis involves most glomeruli and consists of moderate hypertrophy and hyperplasia of glomerular cells with occlusion of many of the capillaries (Fig. 2); +++ glomerulonephritis involves virtually all glomeruli with marked hypertrophy and hyperplasia of endothelial and epithelial cells leading to overall glomerular enlargement, avascularity and obliteration of the capsular space with or without evidence of glomerular hemorrhage (Fig. 3); and ++++ includes the previous changes plus evidence of focal glomerular necrosis. Arterial changes are graded from + to ++++, depending upon amount of intimal reaction, vascular necrosis, and inflammatory exudate, with + lesions showing only swelling of intima and slight focal inflammatory exudate and ++++ lesions showing almost complete fibrinoid necrosis of vessel walls and extensive inflammatory reaction. Endocarditis usually consisted of mononuclear infiltration of mural or valvular endocardium, proliferation of endocardial lining cells and occasional foci of subendocardial fibrinoid necrosis (Fig. 4). Glomerular alterations have been used as a basis for correlation of morphologic and serologic developments in these rabbits. The characteristic proliferative change in glomeruli is the most frequent morphologic manifestation of serum sickness. Since serum sickness affects the kidneys more or less generally, the study of at least 1 complete cross section of each kidney would seem to afford an ade-

TABLE I. Histologic and Serologic Changes in Rabbits Injected with 250 mg BSA per kg.

Rabbit No.	Day of antigen elimination	Lesions of serum sickness: Glomerulonephritis	Arteritis	Endocarditis and valvulitis	Decrease in serum complement (% CH_{50} units)	Maximum BSA in complex form*
5271	11	++	++++	+	67	1.4
5276	"	++++	++++	+	65	1.7
5150	12	+++	+	++		1.3
5261	"	++	++++	++		1.9
5270	"	++++	++++	++	78	1.0
5277	"	++++	0	+		.9
5153	13	++	0	++		1.5
5258	"	++	++	+	78	1.4
5263	"	++++	+	++	71	2.1
5264	14	0	+	0	66	1.5
5152	15	0	0	0		1.7
5259	"	++	0	0	72	1.5
5262	"	++++	++	++	83	1.9
5265	"	0	0	0	66	1.6
5272	"	+++	0	0	61	1.6
5273	"	++	0	0		.4
5274	"	+++	0	0	71	1.2
5154	17	+	0	0		.5
5149	18	0	0	0		1.7
5268	†	0	0	0		.0
5269	†	0	0	0		1.1
5275	†	0	0	0		.7
5151	‡	++	0	++	0	3.5
5260	§	+	0	0	0	.5
5266	§	++	0	0	0	.7
5267	§	0	0	0	0	1.7

* % of total BSA inj.

† Antigen being eliminated by immune mechanism, but not completely removed at time of sacrifice, day 16.

‡ No detectable immune elimination of antigen, sacrificed day 14.

§ *Idem*, sacrificed day 16.

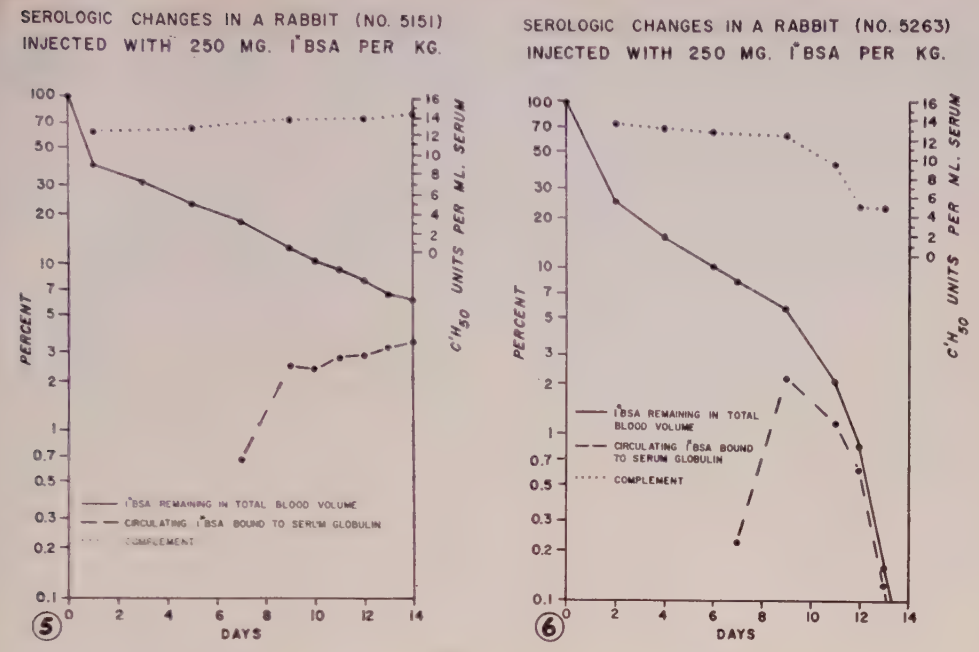

FIG. 5 & 6.

quate histologic sample of renal tissue. Arterial and to a lesser extent endocardial lesions, tend to be focal and their incidence is difficult to quantitate even on the basis of numerous histologic sections. However, in spite of this limitation, the severity of renal, vascular, and endocardial lesions recorded in the table are in fair agreement.

Results. Both histological and serological results are given in the accompanying Table. As reported (13), the most severe renal and arterial lesions developed in rabbits eliminating antigen earliest. However, of the 4 rabbits showing no immune antigen elimination, 2 (5151 and 5266) had significant glomerulitis (Fig. 2) and 1 (5151) had an extensive endocarditis and valvulitis (Fig. 4).

Since all animals (with the exception of rabbit 5268) had considerable amounts of circulating I*BSA-anti BSA complexes, no correlation could be made between presence or absence of circulating complexes in the sera and development of lesions of serum sickness. However, the animals possessing the higher levels of complexes in their sera had a tendency to develop the more severe lesions (Table). Antigen-antibody complexes often appeared in the sera a day or more before there was an increase in rate of elimination of the antigen, and in some animals they continued to circulate in the blood for a considerable time without an associated immune elimination of the antigen. The 4 rabbits which showed no immune elimination of antigen possessed antigen-antibody complexes in their sera for periods of 6 to 8 days. Forty %, or more, of the circulating I*BSA present in these rabbits was free, and not in the form of antigen-antibody complexes. Fig. 5 gives data obtained from serum of rabbit 5151, which had no immune elimination of the I*BSA, but at one time had over 50% of the circulating I*BSA bound to globulin (antibody), and developed glomerulonephritis and endocarditis. Fig. 6 shows data from serum of rabbit 5263, which had a complete immune elimination of antigen on day 13. This rabbit had a considerable portion of antigen bound in the form of antigen-antibody complexes during the immune elimination, and on autopsy it had glomerulonephritis, arteritis, and endocarditis.

The complement levels declined in all animals showing an immune elimination of antigen (Table). This decline in complement accompanied, but never preceded, immune

elimination of antigen, even when a large amount of antigen-antibody complex was present in the sera (Fig. 5 and 6). However, this utilization of complement was not correlated with development of serum sickness in all animals. Rabbit 5265 showed a fall in complement without the appearance of lesions, on observation similar to that of Moll and Hawn(9). Rabbits 5151 and 5266 developed lesions without showing a fall in serum complement levels.

Discussion. On the basis of present evidence, it would seem that while antigen-antibody complexes are probably involved in pathogenesis of serum sickness(6,13), they do not, by themselves, always induce typical lesions of this disease. Almost all rabbits respond to large injections of BSA by making antibodies capable of forming complexes with the antigen, but all animals do not develop lesions. This failure of complexes *per se* to induce lesions is in line with the difficulty experienced in producing the typical morphologic picture of serum sickness by prolonged perfusions of rabbits with soluble antigen-antibody complexes in amounts comparable to those found in active serum sickness(13). In mice repeated large injections of BSA-rabbit anti BSA complexes have given more consistent lesions of kidneys and cardiovascular systems as reported by Benacerraf. Germuth and Pollack have been able to induce arterial and to a lesser degree renal lesions by passive transfer of anti-BSA to rabbits previously injected with the specific antigen(14). It was postulated that these lesions may have been the result of complexes which formed when the transferred antibody reacted with excess circulating antigen. However, the complexes formed under these conditions may differ greatly from those formed in active serum sickness, *e.g.*, the antibody transferred by Germuth and Pollack was obtained from hyperimmune rabbits, whereas, the antibody in active serum sickness usually results from a primary antibody response. The fact that the kind of complexes formed in active immunization varies from animal to animal(7), probably as a result of differences in quantity and quality of antibody formed, may in part explain the variability in lesions among animals. It also may be that antigen-antibody complexes are only one of several elements essential in pathogenesis of serum sickness.

Those animals in which complexes form and are eliminated earliest tend to have the most severe lesions of serum sickness. However, this relationship is not absolute and in 3 animals with relatively early elimination of antigen, there were few, if any, lesions; and in 2 animals not eliminating the complexes, there were lesions. The demonstration of circulating complexes was the only serologic evidence of an immune response in these latter animals. In a few instances, Hawn and Janeway(4) observed serum sickness in humans and rabbits in the absence of an immune elimination of antigen from the blood or other evidence of an antibody response. It may have been that in these cases the host had responded with antibodies capable of forming pathogenic complexes which were not eliminated from the blood.

An insufficient production of antibody might be responsible for the ability of antigen-antibody complexes to exist, in the circulation, without being rapidly eliminated. It appears that the first antibody synthesized combines with the circulating antigen in great antigen excess and that the resulting complexes are not rapidly eliminated. This is evidenced by the presence of a considerable level of complexes in the circulation for several days prior to immune elimination of antigen in most of the rabbits. As additional antibody is synthesized, it probably combines with both the free antigen and the antigen in the complexes. A continuation of antibody synthesis would result in formation of larger complexes and, possibly, even aggregates of complexes. When complexes or aggregates of complexes reach a certain size they are probably removed from the circulation by phagocytic cells(15). In the rabbits, which possessed circulating complexes, but did not have an immune elimination of antigen, total amount of antibody produced might have been too small to permit completion of this process.

Serum complement did not appear to play a

consistent role in pathogenesis of serum sickness in the present experiment. Reduction of circulating complement has been associated with immune elimination of antigen and occurrence of glomerulonephritis in animals injected with large amounts of foreign protein (8,9). In our animals, too, complement levels declined during immune elimination of antigen. Initiation of the drop in level of circulating complement always coincided with the beginning of immune elimination of antigen. That the fall in complement levels depends upon more than interaction between antigen and antibody is evident in those animals with circulating complexes, but without a fall in complement or an immune elimination of antigen. It cannot be determined by the present data whether immune elimination might cause fixation of complement or whether fixation of complement, to circulating complexes, might result in immune elimination. Whatever the relationship between complexes and complement, utilization of serum complement was not always correlated with development of lesions of serum sickness. One rabbit showing a definite fall in complement, associated with immune elimination of antigen, had no detectable morphologic lesions. On the other hand, the development of lesions occurred without measurable participation of serum complement in 2 rabbits.

Summary. Neither the level of antigen-antibody complexes in the serum nor amount of complement utilized during the responses of rabbits to large injections of BSA could be correlated consistently with either the development or severity of the lesions of serum sickness. Almost all rabbits responded to large injections of BSA by making antibodies capable of forming complexes with circulating antigen yet only ⅔ of them developed significant lesions. On the whole those animals making the earliest responses and eliminating the complexes most rapidly had the most severe lesions; but some animals in which complexes were not eliminated rapidly from the blood also developed lesions. Likewise complement did not appear to be utilized in some rabbits which developed lesions and in some rabbits showing a fall in complement no lesions were found. Thus, it would appear that either there are other factors in addition to complexes and complement which are essential in the pathogenesis of serum sickness or that the complexes vary in their properties from animal to animal and that some of these complexes are more pathogenic than others.

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Lethal Effect of Certain Benzimidazoles and Benzenes on Early Chick Embryo.*† (24305)

UNABELLE BOGGS BLACKWOOD‡ AND MARY S. SHORB
Department of Poultry Husbandry, University of Maryland, College Park

Little is known of the biochemical mechanism(s) interrupted by the benzimidazoles. Unsubstituted benzimidazole inhibition in yeast is reversed by adenine and guanine(1), but the 2, 5-alkylbenzimidazoles are only partially reversed by Vit. B_{12}(2,3). A characteristic order of inhibitory activity has been found in several biological systems(2,4-8) in which 2-ethyl-5-methylbenzimidazole is considerably more active than either the 2, 5-dimethyl or 5, 6-dimethyl analogues and all alkyl analogues are more active than unsubstituted benzimidazole. However, increasing the alkyl chain past 2 carbons in the 2 position does not further increase inhibition of influenza virus multiplication(4). Dimethyldiaminobenzene (a degradation product of 5, 6-dimethylbenzimidazole and B_{12}) and its analogue, dichlorodiaminobenzene, also have characteristic orders of inhibition depending upon the Vit. B_{12} requirement of the system being inhibited. Dichlorodiamine inhibits synthesis of Vit. B_{12} and riboflavin in microorganisms(9) and inhibits the growth of non-requiring-B_{12} and/or riboflavin microorganisms but has no effect on organisms (including mice) which require these vitamins(10). The opposite is true of dimethyldiamine; it reverses the dichlorodiamine or dibromodiamine in microorganisms able to synthesze the vitamins(3,10) and inhibits those organisms which require an exogenous source of Vit. B_{12}(11). However, in virus inhibition the dichlorodiamine is only slightly more effective than the dimethyldiamine and the 2 are equally toxic to the chorioallantoic membrane(12).

In our study the order of lethality of the benzimidazoles to the early chick embryo is identical to that found in virus inhibition and the order of the benzenes is the same as that found in organisms able to synthesize Vit. B_{12}.

Materials and methods. In addition to previously mentioned benzenes, the benzimidazole analogues; 5, 6-dimethyl, 2, 5-dimethyl, 2-ethyl-5-methyl and 2-hepta-5-methyl; and methylamide (an inhibitor of Vit. B_{12} in microorganisms)(13,14) were studied as lethal agents in early chick embryo. The effect of Vit. B_{12} on 3 of the compounds was tested.

The benzimidazoles and benzenes were completely dissolved in dilute HCl (0.06-0.13 N), 10 mg per ml, then diluted with double distilled water (used throughout) to the concentration injected into the egg (Table I). Methylamide and Vit. B_{12} were dissolved in water. The benzimidazoles and Vit. B_{12} were autoclaved for 10-15 min. at 15 lb pressure before injection, but the benzenes, because of their unstable nature, were autoclaved dry and dissolved in sterile HCl.

Eggs from 2 strains of White Leghorns, Mt. Hope and random bred, were used. The eggs were never stored for more than 3 days previous to injections. Groups of 10 to 25 eggs were used per treatment. Each egg was dabbed with tincture of iodine, wiped with 70% ethanol, punctured with a mechanical egg shell puncher, and injected through the air cell into the albumen with a sterile #27 needle mounted on a 1 ml syringe with marked plunger. Each egg received 0.1 ml solution. The eggs were immediately incubated in Jamesway forcedraft incubators at 99-100°F. They were candled the fifth day of incubation and daily thereafter until the 18th day. Each infertile or dead embryonated egg was removed and opened for examination. The percent dead of fertile eggs per experimental

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‡ U. S. Public Health Predoctorate Research Fellow.

TABLE I. Lethal Effect of Several Inhibitors on Chick Embryos following Injection into Eggs Prior to Incubation.

No.	Compound	μM/egg	No. of groups	Total fertile eggs	% dead 18 days	Lethal index*
	None	—	4	132	5.4 (3–10)	—
	HCl (.06-.13 N)	.1 ml	11	142	7.9 (0–22)	—
1.	Methylamide	13.6	1	17	0.0	.0
	"	16.9	1	19	26.	1.5
	"	169.5	1	15	13.	.1
2.	1,2-Dimethyl-4,5-diaminobenzene	3.7	1	13	8.	2.1
	"	5.5	1	15	20.	3.6
	"	7.3	1	15	20.	2.7
3.	1,2-Dichloro-4,5-diaminobenzene	1.99	3	48	14.3 (14–15)	7.2
	"	2.8	4	60	33.7 (13–67)	11.9
4.	5,6-Dimethylbenzimidazole	3.4	1	13	15.	4.4
	"	4.8	1	13	23.	4.8
	"	6.8	1	14	14.	2.0
5.	2,5-Dimethylbenzimidazole	1.7 (1.4–2)	3	50	23.3 (20–25)	14.0
	"	3.1 (2.7–3.4)	5	60	24.0 (6–40)	7.7
	"	5.6 (4.8–6.8)	3	36	13.3 (0–30)	2.3
6.	2-Hepta-5-methylbenzimidazole	1.3	2	33	20.5 (12–29)	15.8
	"	2.2	3	51	29.3 (26–31)	13.4
	"	3.0	3	46	51.0 (25–69)	16.8
	Oct.-Nov.; random bred, white leghorn					
7.	2-Ethyl-5-methylbenzimidazole	2.19	2	34	29.0 (25–33)	13.2
	"	3.12	2	38	37.5 (31–44)	12.0
	Feb.-March; Mt. Hope, white leghorn					
	"	.62	2	26	31.0 (20–22)	35.5
	"	1.25	2	23	40.0 (30–50)	32.0
	"	2.19	2	20	65.0 (60–70)	29.7
	"	3.12	3	37	59.0 (55–64)	19.0

* Lethal index expresses % dead/μMol inhibitor. ( ) = Range of individual group results.

group was, in each case, averaged. For comparison of inhibitors a lethal index (indicating dose effectiveness) was devised by dividing percent dead embryos by the number of micromoles of inhibitor per egg. Vit. B_{12} and various normalities of HCl required to dissolve the highest concentration of inhibitors served for control injections.

Results. Table I shows that injection of dilute HCl produced only slightly greater mortality than found in noninjected controls. Thus the HCl in which inhibitors were dissolved cannot be considered to be a significant factor in producing the mortality found in inhibitor groups. When dose size of compounds, particularly 3, 6, and 7, was increased, mortality also increased but the lethal index decreased or increased depending on the in-

hibitors (Table I). The relationship of lethal index to change in dose size also varied with other inhibitors (Table I), thus making it difficult to compare the compounds as lethal agents. However, by averaging the lethal indices the compounds are rated in the following order given with their respective averaged values: methylamide, 0.5; 1, 2-dimethyl-4, 5-diaminobenzene, 2.8; 5, 6-dimethylbenzimidazole, 3.7; 2, 5-dimethylbenzimidazole, 8.0; 1, 2-dichloro-4, 5-diaminobenzene, 9.5; 2-ethyl-5-methylbenzimidazole, 12.6 (Oct.-Nov., random bred); 2-hepta-5-methylbenzimidazole, 15.5; and 2-ethyl-5-methylbenzimidazole, 29.0 (Feb.-Mar., Mt. Hope).

The lethal effect of 2-ethyl-5-methylbenzimidazole, but that of no other inhibitor, could be correlated with seasonal or genetic differences or both. This inhibitor had considerably greater effect when injected into Mt. Hope eggs during Feb. and Mar. than when injected into random bred eggs during Oct. and Nov. (Table I).

Vit. B_{12} (100-500 μg) injected alone produced an average of 3.3% mortality with a range of 0-10%; these values do not differ from those found in noninjected controls (Table I). B_{12} is, therefore, nonlethal at these levels. B_{12} at high levels had no effect on mortality produced by 2-hepta-5-methylbenzimidazole and 1, 2-dichloro-4, 5-diaminobenzene, but it decreased mortality produced by the 2-ethyl-5-methyl analogue under conditions resulting in greater lethality of this compound; *i.e.*, when 100 μg B_{12} (but not 50 μg) with 2.19 mcMol inhibitor were injected during Feb. and Mar. into Mt. Hope eggs, the mortality decreased from 65 to 40%; also, 250 μg B_{12} with 3.12 mcMol inhibitor reduced mortality from 56 to 33%. However, B_{12} had no effect when the same combinations of B_{12} and inhibitor were injected into random bred eggs during Oct. and Nov. Unpublished data on the 1½-day-incubated egg gave essentially the same results.

Discussion. The seasonal and/or genetic variation in lethality of 2-ethyl-5-methylbenzimidazole and the reversal by B_{12} may be due to seasonal and genetic dependent variation in deposition of Vit. B_{12} in the yolk by the hen. A normal seasonal variation in B_{12} deposition has been reported(15) in which there is greater deposition in the fall than in winter and spring. This is in accordance with the seasonal variation found in our investigatons. The large amounts of B_{12} required to affect a response could be due to binding of B_{12} by the egg albumen, but further work is needed to clarify this point. The data presented here are not adequate to determine whether the variation found with 2-ethyl-5-methylbenzimidazole is predominately related to seasonal or genetic differences or both. However, Landauer(16) found lethal and teratogenic action of compounds in chick embryos to be related to both seasonal and genetic differences.

The failure of 2-hepta-5-methylbenzimidazole to show greater lethality than 2-ethyl-5-methylbenzimidazole makes the relative order of activity of benzimidazoles exactly the same as that found with the virus multiplication inhibitory work(4); this would seem to indicate that the same mechanisms are interrupted in the early embryo as in virus multiplication. The relative lethality of the benzenes, however, did not agree with the virus work, nor with the toxicity to the chorioallantoic membrane taken from 10-day embryos(12), nor inhibition of B_{12} requiring microorganisms (11), but did agree with the inhibition of microorganisms which are able to synthesize Vit. B_{12} and riboflavin(9,10). The implication is, thus, that the early embryo synthesizes Vit. B_{12} and/or riboflavin. Work with B_{12} deficient eggs(17) and B_{12} levels in the yolk and embryonic tissue(15) indicate that the chick embryo either does not require B_{12} during the first week of incubation or is able to synthesize it. It should be noted, in this connection, that the 3 compounds which are natural moieties of Vitamin B_{12}, methylamide, 1, 2-dimethyl-4, 5-diaminobenzene, and 5, 6-dimethylbenzimidazole, were the least lethal of the compounds tested.

Summary. (1) The lethality of 7 inhibitors structurally related to Vit. B_{12} was determined in chick embryos by injecting several doses of each of them into the albumen prior to incubation. (2) Vit. B_{12} alone was nonlethal and failed to influence the lethality of 2-hepta-5-methylbenzimidazole and 1, 2-dichloro-4, 5-diaminobenzene. (3) Lethality

of 2-ethyl-5-methylbenzimidazole as well as its partial reversal by high levels of Vit. B_{12} varied with seasonal and/or genetic differences. (4) The relative order of lethality of benzimidazoles was the same as in virus inhibitory work, while that of benzenes was the same as the inhibition of microorganisms which synthesize B_{12} and/or riboflavin. (5) The 3 inhibitors which are natural moieties of Vit. B_{12}, methylamide, 1, 2-dimethyl-4, 5-diaminobenzene, and 5, 6-dimethylbenzimidazole, were the least lethal of the compounds tested. The implications of these findings are discussed.

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Latex-Leptospiral Agglutination Test. (24306)

THELMA F. MURASCHI (Introduced by John F. Kent)
(With technical assistance of Marjorie Hulett and Anne Leone)
Division of Laboratories and Research, New York State Department of Health, Albany

The successful use of sensitized latex particles(1) in an agglutination test for rheumatoid arthritis(2) suggested applications in the serodiagnosis of leptospirosis. Latex-leptospiral suspensions were found to form macroscopic specific aggregates with hyperimmune rabbit serum, and the study was extended to the human infection. This report gives details of the technic, and compares results of the latex agglutination test with those obtained in standard agglutination-lysis and microscopic agglutination tests.

Materials and methods. Polystyrene latex particles (diameter, 0.81 μ, in 11% aqueous suspension)* were diluted 1:10 in distilled water, filtered through Whatman #40 paper to remove any aggregates, then adjusted with water until a further 1:100 dilution in glycine-buffered saline solution† had an optical density of 0.25 $\pm$ 0.01 at 650 mμ. The optical density measurements were made in 12 x 75 mm cuvettes, using the Coleman Jr. Spectrophotometer 6A with adapter 6.106. Leptospiral suspensions were prepared from 7-day-old cultures in modified Korthof's medium with yeast extract(3). The organisms were killed by overnight exposure to formalin, which was added to give a final concentration of 0.5%. They were then concentrated by spinning 20 min. at 13,000 G, washed twice with formalinized (0.5%) 0.85% NaCl solution (FS), and resuspended in about 1 ml FS per 100 ml original culture. The concentrate was homogenized, using a tuberculin syringe with 22 gauge needle, and spun 3 min.

* Kindly provided by the Dow Chemical Co., Midland, Mich.

† One liter of aqueous solution contained 10.0 gm NaCl, 7.31 gm NH_2CH_2COOH, and 3.5 ml NaOH (1 N); pH was 8.2 at 25°C.

TABLE I. Standardization of Latex-Leptospiral Antigen.

Leptospiral suspension added*	Reactivity† with: Antiserum diluted 1: 4‡	8	16	32	64	128	Normal sera 1:100 A	B	C	D	E	Saline solution
2.0	4	4	4	3	3	3	1	1	1	—	—	3
1.5	4	4	3	2	1	2	±	±	±	—	—	2
1.0	4	3	2	1	—	—	—	—	—	—	—	—
.8	4	3	2	1	±	—	—	—	—	—	—	—
.6§	4	4	3	2	1§	—	—	—	—	—	—	—
.4	4	4	3	2	1	2	—	±	±	—	—	2
.2	4	3	3	2	2	2	1	1	1	—	—	3
.1	3	2	2	2	1	1	1	1	1	—	—	3

* ml/0.1 ml standardized latex suspension.
† Numerical values represent degree of agglutination; negative sign (—), no reaction.
‡ $\times 10^2$.
§ Optimal volume for diagnostic tests.

at 125 G to sediment any large aggregates. The supernate was adjusted finally with FS to an optical density of 0.60 ± 0.01 at 420 mμ. The optimal concentration for diagnostic tests was determined by adding increasing volumes (0.1 – 2.0 ml) to 0.1 ml aliquants of standardized latex suspension and diluting to 10.0 ml with glycine buffered saline solution (GBS). The resulting antigens were tested against human antisera diluted serially in GBS. Equal volumes (1.0 ml) of serum and antigen were mixed in 12 x 75 mm tubes, the tubes corked, and the contents incubated 90 min. at 56°C. Following incubation the corks were removed and the tubes spun 3 min. at 1000 G. The contents were then examined by transmitted light against a dull black background, the aggregates being kept suspended by briskly tapping the tubes with the fingertips. Degrees of agglutination were recorded in the conventional notation (± to 4+). Table I gives results of a representative antigen standardization. The given serum dilutions take into consideration the final 2-fold dilution by antigen. Sera of healthy individuals, who served as controls, were tested at the lowest dilution (1:100) used for diagnostic tests. Excess or deficiency of leptospiral suspension predisposed to spontaneous agglutination, as well as to pseudo-reactions with normal sera. However, there was an intermediate specific zone in which reactivity varied inversely with volume of leptospiral suspension. Optimal volume was the smallest in this range; ordinarily it approximated 0.5 ml. Larger volumes of latex-leptospiral antigen prepared for diagnostic tests contained the components in the same proportion. Freshly-prepared latex-leptospiral antigen was visibly particulate, but appeared less definitely so when diluted with test serum. Preliminary "aging" reduced the particulate appearance, the antigen becoming stabilized over a period of 10-14 days, and remaining constant in reactivity for at least 2 years. The pool of leptospirae used in preparing latex antigen for qualitative screen tests contained *L. icterohemorrhagiae, L. pomona,* and *L. canicola* suspensions in equal volume. These were the serotypes most frequently encountered in our own serodiagnostic practice, and as a pool exhibited a high degree of cross reactivity with antisera representing 17 other serotypes. Sera used in evaluating sensitivity of the tests fell into 2 categories; 1) paired specimens from patients with leptospiral infections verified by isolation and identification of the inciting agent, and 2) single specimens from patients with clinical leptospirosis confirmed by serologic tests. The former were obtained through Miss C. McComb‡ from Dr. J. T. Tonge§, who provided ancillary data on the inciting serotypes, days of illness, and agglutination-lysis titers. The latter were encountered in serodiagnostic practice, and were subjected to microscopic agglutination tests by Dr. N. Hirschberg.‖ The indispensable and generous

‡ Harvard School of Public Health, Boston, Mass.

§ Dept. of Health and Public Affairs, Brisbane, Australia.

‖ State Laboratory of Hygiene, Raleigh, N. C.

TABLE II. Comparison of Latex-Agglutination (L-A) Serum Reactions with Agglutination-Lysis (A-L) or Microscopic-Agglutination (M-A) Reactions in Human Leptospirosis.

Leptospira isolated	Days ill	Serum reactions: Quantitative* A-L	Quantitative* L-A	L-A screen†	Leptospira isolated‡	Days ill	Serum reactions: Quantitative* A-L	Quantitative* L-A	L-A screen†
Australis A	2	—	—	—	"Kremastos"	2			—
	39	300	—	—		32			R
	3	—	—	—	"	3			—
	38	3,000	1,600	R		57	100		R
	5	—		—	*hyos*	4			—
	82	1,000	1,600	—(R)		52	300	6,400	R
	4		400	—(R)	*pomona*§	8	—	100	R
	86	100	200	R (R)		21	1,000	3,200	R
	2	—	400	—(R)	" §	6	—	200	—
	83	300	100	—(R)		22	1,000	3,200	R
	4	—			" §	6	—	—	—
	25	3,000	12,800			22	1,000	1,600	R
					"	2	—	—	R
	45	3,000	12,800	R		45	300	800	R
	3	—	—	—	"	2	—	—	—
	75	300	—	R		40	100	800	R
Australis B	7			—	"Robinson"	3	—		—
	44	100	1,600	R		21	10		R
	3	—	100	—			Quantitative M-A		
	22	300	25,600	R					
	3	—		—	*bataviae*§				
	72	1,000		—		41	1,600	3,200	R
	3	—		—	*canicola*§				
	41	300	200	R		32	6,400	12,800	R
	5	—	400	—	*ictero.*§				
	52	1,000	3,200	R		44	6,400	12,800	R
	1	—		—	*pomona*§				
	46	300	200	R		77	3,200	6,400	R
	4	—	400	—(—)					
	53	100	400	—(R)					
	3	—		—					
	67	100	400	R					
	4	—	800	—(R)					
	23	300	800	R (R)					

* Titers are reciprocals of highest dilutions reacting (2+ to 4+) with respective homologous antigens.

† Reactive (R = 2+ to 4+) or non-reactive (negative sign = ± to 1+) at a final 1:100 dilution with an antigen in which leptospiral component was pooled *L. ictero., L. pomona,* and *L. canicola.* Results in parentheses represent repeat tests in which *L. canicola* was replaced with homologous leptospirae.

‡ Except in cases designated § (q.v.).

§ Leptospirae not isolated; diagnosis based on serologic tests.

contributions of these collaborators are gratefully acknowledged. The control sera used in evaluating the specificity of the latex tests were from healthy individuals, and from patients with syphilis. All were stored at –20°C. Serial 2-fold dilutions in GBS, beginning at 1:50, were prepared for quantitative tests with homologous antigen, and a 1:50 dilution served for qualitative screen tests with pooled antigen. The testing procedure has been described under antigen standardization. Sera were designated reactive (2+ to 4+ agglutination) or non-reactive (– to 1+ agglutination). Titer was expressed as the reciprocal of the highest reactive dilution; numerical value, as in the agglutination-lysis and micro-

scopic-agglutination tests, took into account the final 2-fold dilution of serum by antigen.

Results. Results obtained in quantitative latex-agglutination (L-A) tests of human sera are compared in Table II with those of agglutination-lysis (A-L) and microscopic-agglutination (M-A) tests conducted in other laboratories; 30 individuals are represented. L-A titers with few exceptions were higher than those of standard A-L or M-A tests, the greater sensitivity of the test in some instances making the difference between reactivity and non-reactivity, and revealing antibody before it was detectable by standard procedures. A qualitative L-A screen test in which the antigen component was pooled *L. icterohemorrhagiae, L. pomona,* and *L. canicola* was evaluated with the same sera. Although the component leptospirae were present at one-third the optimal concentration for quantitative tests, reactions(R) were observed in all 8 cases of homologous infection. Reactivity was also observed in 16 (76%) of 21 patients whose infections were caused by other leptospiral serotypes such as *australis* A or B, "Kremastos"(4), *hyos,* "Robinson"(4), and *bataviae.* The screen test failed to detect antibody in 5 of 16 cases of *australis* A or B infection. The initial negative result, however, was reversed in 3 instances by replacing the *L. canicola* component of the antigen with homologous leptospirae. There was insufficient serum to retest the others. The specificity of the latex agglutination reaction was examined in tests of 162 healthy and 14 syphilitic individuals. Three of the former reacted, but only at the lowest final dilution (1:100) tested; the remainder were seronegative. The latex-agglutination tests thus compared favorably in sensitivity and specificity with standard agglutination-lysis and microscopic-agglutination tests. They had additional advantages in simplicity and rapidity of performance, and in the use of a stable, noninfectious antigen.

Summary. The reaction of leptospiral antisera with an antigen composed of polystyrene latex particles and formalin-killed leptospirae provides the basis for a simple, macroscopic tube agglutination test for leptospirosis.

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Gas-Liquid Chromatography of Highly Unsaturated Fatty Acid Methyl Esters.* (24307)

W. STOFFEL, W. INSULL, JR., AND E. H. AHRENS, JR.
The Rockefeller Institute, N. Y. City

Biochemical studies of fatty acid metabolism have been given great impetus by the development of gas-liquid chromatography (GLC) by James and Martin(1). This method permits microgram mixtures of methyl esters of fatty acids to be resolved when these esters are volatilized and distributed between a moving carrier gas (nitrogen or argon) and a stationary liquid phase. At the high temperatures (up to 200°C or more) required for volatilization, degradation of saturated fatty acid esters has not been encountered, but the possibility that the double bond structure of highly *un*saturated esters might be altered under these conditions has not been ruled out. In view of growing interest in the biologic role of unsaturated fatty acids as integral parts of certain enzymes(2) and in human nutrition(3), it seemed imperative to investigate the stability of these acids during

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TABLE I. Chemical Structure and GLC Characteristics on 2 Stationary Phases of Unsaturated Fatty Acids of Natural Origin.*

Fatty acid methyl esters	Position of double bonds†	Apparent retention vol relative to stearic acid methyl ester = 1.00 at 197°C: Apiezon M	Reoplex 400
C_{16} saturated		.41	.53
monoenoic	8	.37	.60
	9	.37	.60
dienoic	6,9	.36	.68
	9,12	.33	.74
trienoic	6,9,12	.32	.84
tetraenoic	6,9,12,15	.31	1.04
C_{18} saturated		1.00	1.00
monoenoic	9	.87	1.12
dienoic	9,12	.76	1.35
	6,9	.76	1.35
trienoic	9,12,15	.77	1.58
tetraenoic	6,9,12,15	.72	1.84
C_{20} saturated		2.44	1.90
dienoic	‡	1.88	2.05
trienoic	‡	1.64	2.29
tetraenoic	8,11,14,17	1.46	2.83
pentaenoic	5,8,11,14,17	1.46	3.60
C_{22} saturated		5.95	3.60
pentaenoic	7,10,13,16,19	not done	6.60
hexaenoic	4,7,10,13,16,19	3.00	7.34

* All acids except the C_{22} group were isolated from menhaden body oil and characterized as previously described(4). C_{22} acids were generously supplied by Prof. E. Klenk, Cologne, Germany, having been isolated from herring oil.

† Numbered from carboxyl carbon.

‡ Double bond structure not yet clarified.

analysis by GLC. The present report demonstrates that highly unsaturated long chain aliphatic fatty acid methyl esters are exceedingly stable when chromatographed under James and Martin's conditions. In addition, GLC analytical data needed for identification of 17 of these esters in mixtures of biologic origin are presented.

Materials. The unsaturated long chain acids listed in Table I were isolated and their structures identified by procedures described elsewhere(4). For the most part the acids were derived from a specially prepared sample of menhaden body oil† used by us in human nutritional studies(5).

† Generously supplied by Dr. T. M. Miller, Marine Chemurgics, Morehead City, N. C.

Methods. GLC was carried out essentially as described by James and Martin(1) using the ionization chamber detector of Lovelock and James(6) vapor-jacketed at 197°. The mobile phase, argon, was applied at a pressure of 67 cm Hg. Each 4-foot column was packed with 5 g of acid-washed, alkali-treated Celite 545 (mesh 140-200). Two stationary phases were studied, the non-polar hydrocarbon vacuum grease Apiezon M(1) and the polar polyester Reoplex 400(7). The Apiezon column contained 0.8 g Apiezon; with a flow rate of 52 ml A/min this column had an efficiency of 3500 plates at methyl stearate (retention time of 60 minutes). The Reoplex column contained 2 g of Reoplex 400 and had a column efficiency of 2060 plates at methyl stearate (retention time of 21 minutes, flow rate 52 ml A/min). Both columns were conditioned at 197° by flushing with nitrogen applied at 67 cm Hg pressure for at least 3 days before analytical use. All relative retention volumes were calculated from apparent retention times (= time from air peak to center of symmetrical elution curve). Esters were recovered after chromatography by leading the effluent gas from the detector into a glass tube loosely packed with defatted cotton moistened with absolute methanol. The decomposition of organic vapor passing through this detector has been estimated to be about one in 10^9 molecules(8).

Stability studies. A. Criteria. Our stability studies have been limited to date to GLC on the non-polar stationary phase, Apiezon M. For proof of complete stability we demanded: quantitative recovery of acids applied to the column, no change in retention volume of methyl esters repeatedly re-chromatographed, no production of *trans* double bonds as shown by infrared spectroscopy(9), no formation of conjugated double bonds and no alteration in specific extinctions in the ultraviolet range after isomerization in alkali(10), and, finally, no alteration in double bond positions as shown by identification of fragments produced by ozonolytic degradation(4).

B. Results. The most highly unsaturated ester in each chain length group (Table I) was studied. Quantitative recovery of 2-10

mg of each of the 4 esters was demonstrated by microgravimetric analysis of the products applied to the column and recovered from the effluent trap. Test runs varied from 1-3 hours. Over these periods there was no detectable "bleeding" of the Apiezon M stationary phase, as shown by absence of weighable residue in traps applied to the uncharged column for 3 hours. These gravimetric analyses were considered accurate to 1% or better (with samples of 2 mg and balance sensitivity of ± 0.01 mg(11)).

Relative retention volumes of the 4 esters were determined individually by applying small charges (1/16 μl) of each together with the respective saturated homolog and methyl stearate. The unsaturated esters were trapped and re-run. There was no change in relative retention volume of each ester after 2 re-runs.

Infrared and ultraviolet spectra of each of the 4 esters, before and after GLC on Apiezon M, were almost identical. Infrared patterns showed no evidence of *cis*⟶*trans* isomerization. Ultraviolet spectra of esters in concentrated solutions (1 mg/2 ml methanol) showed only slight increases in conjugated double bonds: the C_{16}-tetraenoic fraction showed an increase of di-, tri- and tetraene conjugation of less than 1%; the C_{20}-pentaenoic acid conjugation rose from 1 to 2% in the diene region only; the C_{22}-hexaenoic acid showed an increase in diene conjugation from 6 (before) to 10% (after GLC) without conjugation in the higher polyene regions. After isomerization in alkali(10), the 4 chromatographed esters showed ultraviolet spectra which were qualitatively and quantitatively identical to those of the original esters.

Two polyenoic acids were degraded by micro-oxidative ozonolysis(4) before and after GLC on Apiezon M. The dicarboxylic acids produced were methylated(12) and chromatographed on Apiezon M at 78°. In the case of both samples of C_{18}-tetraenoic acid, adipic and malonic acid di-methyl esters were identified as sole dicarboxylic acids, while with the C_{20}-pentaenoic acid only glutaric and malonic acid di-methyl esters were found before and after GLC. These studies showed that the methylene-interrupted double bond structure of the 2 acids (divinyl methane rhythm) was not altered by GLC, and that the positions of the double bonds along the chains had not shifted.

Retention volumes relative to methyl stearate (= 1.00) are presented in Table I for all polyenes studied. On Apiezon M the unsaturated esters precede their saturated homologs through the column, but the separations of the individual polyenes from each other are not always clean. On Reoplex 400, on the other hand, the polyenes follow their saturated homologs, as shown by Orr and Callen(7,13), and separations within each chain length group are excellent.

Fig. 1 graphs the logarithms of retention volumes relative to methyl stearate (= 0) when Reoplex 400 is the stationary phase. The heavy diagonal line shows linear relationship with molecular weight in the case of the saturated homologs. Logarithms of relative retention volumes of unsaturated acid ester groups are shown in dashed lines. Five points deserve comment: 1) relationship between these logarithmic values and number of double bonds in each chain length group approaches linearity; 2) even-numbered unsaturated acids overlie the next higher odd-numbered and branched-chain acids; 3) there are overlaps between C_{16}-tetraene and normal C_{18}-saturate, between C_{18}-tetraene and normal C_{20}-saturate, between C_{20}-pentaene and normal C_{22}-saturate. 4) C_{20}-monoene would not be expected to separate from C_{20}-saturate; 5) the only isomers separated under these conditions were the two C_{16}-dienes.

The fact that both non-polar and polar stationary phases fail to accomplish complete separations of the acids studied here warns against reliance upon any single stationary phase for complete definition of the fatty acid composition of biological mixtures. However, the different chromatographic characteristics of the two phases are complementary and can be used to advantage. In this laboratory complex mixtures have been advantageously resolved by preparative runs (10 μl) on Apiezon M with trapping of effluent fractions between successive even-numbered esters. Analytical runs of these fractions (¼-1 μl) on Reoplex

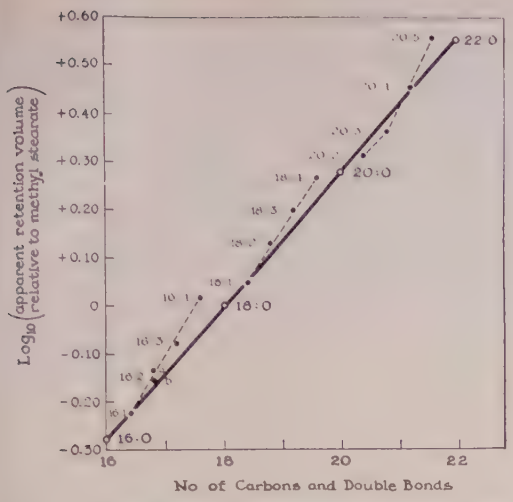

FIG. 1. Logarithms of retention volumes of long chain fatty acid methyl esters relative to methyl stearate (= 0); GLC at 197° on Reoplex 400 as stationary phase. Saturated normal homologs are shown by diagonal solid line. Groups of unsaturated acids are plotted in dashed lines. No. after colon indicates double bonds in acids of stated chain length (*i.e.* 16:4 = C_{16}-tetraenoic acid methyl ester). 16:2a = hexadeca-9,12-dienoic, 16:2b = hexadeca-6,9-dienoic acid.

400 serve to separate the mixtures in each. For example, in analysis of menhaden body oil fatty acids the fraction trapped between the trailing ends of the C_{14}- and C_{16}-saturate curves from an Apiezon M column can be collected and applied to a Reoplex 400 column. This mixture resolves cleanly into individual C_{15} and C_{16} acids, and in addition further separations in each group are based on numbers of double bonds. The success of this approach, an application of which has been described elsewhere by us(5), depends upon rigorous proof of stability of polyunsaturated fatty acid esters during GLC on Apiezon M.

Summary. Proof is presented that methyl esters of highly unsaturated long chain fatty acids are not significantly altered in chemical structure during gas-liquid chromatography with the stationary phase Apiezon M at 197°. Relative retention volumes of C_{16}, C_{18}, C_{20} and C_{22} polyenoic acids are listed for 2 stationary phases, non-polar Apiezon M and polar Reoplex 400. A system for rapid total analysis of complex fatty acid mixtures on a submilligram scale is described.

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Inactivation of Amphotericin B, Chlorquinaldol, Gentian Violet and Nystatin by Bile Salts. (24308)

S. STANLEY SCHNEIERSON, DANIEL AMSTERDAM AND M. L. LITTMAN
Department of Microbiology, Mount Sinai Hospital, N. Y. City

Bile salts exert a variety of effects upon the action of different antibiotics(1). They enhance the antimicrobial activity of neomycin and penicillin against certain bacterial species, inactivate polymyxn, ristocetin and vancomycin and produce no effect whatsoever on other antibiotics. The present report is concerned with the effect of these salts upon the

TABLE I. Inactivation of Antimycotic Agents by 1% Bile Salts.

Antimycotic agent	MIC in Basal broth (μg/ml)	MIC in Basal broth + 1% bile salts (μg/ml)	Increased MIC
Amphotericin B	.4	50	125 ×
Chlorquinaldol	6.0	60	10 ×
Gentian violet	.2	25	125 ×
Nystatin	25.0	300	12 ×

activity of such diverse antimycotic agents as amphotericin B, chlorquinaldol, gentian violet and nystatin.

Materials and methods. The basal broth medium contained Trypticase (BBL) 7.5 g, Phytone (BBL) 1.3 g, sodium chloride 2.15 g, dipotassium phosphate 1.05 g, and glucose 10.0 g per liter of distilled water, adjusted to a pH of 7.2. Appropriate dilutions were prepared from the following sterile stock solutions: bile salts (Difco), 200 mg/ml in distilled water; amphotericin B (Fungizone®), 1 mg/ml in distilled water; chlorquinaldol (Steroson®), 5 mg/ml in 5% HCl; gentian violet, 1 mg/ml in distilled water; and nystatin (Mycostatin®), 5 mg/ml in propylene glycol. Bile salt and gentian violet stock solutions were sterilized by autoclaving at 15 lbs for 15 min., the others were prepared aseptically. All determinations of growth inhibition were performed in 5 ml amounts by the tube dilution method, except those involving gentian violet. These were performed in 10 ml amounts since readings of end points in the presence of the intense color imparted by higher concentrations of the dye was facilitated by the use of a greater volume. The standard inoculum employed throughout was 0.1 ml of an 18 hour culture of *Candida albicans* (MSH #005), grown at 37°C with an optical density of 0.18 at 650 mμ on the Lumetron colorimeter. Tubes were incubated at 37°C for 48 hours at which time they were read visually for the presence or absence of growth as indicated by formation of a distinct button of yeast growth at the bottom of each tube.

Results. Table I lists the minimal inhibitory concentrations (MIC) of each of the 4 antimycotic agents under study for the standard inoculum of *C. albicans* in basal broth as compared to the same medium to which bile salts had been added to yield a concentration of 1.0%. As is shown in the table, all were markedly but unequally inactivated by this concentration of bile salts. This is evidenced by the increased MIC of amphotericin B and gentian violet for *C. albicans* of 125 times, for nystatin of 12 times and for chlorquinaldol of 10 times.

To ascertain whether this finding is limited to the particular strain of *C. albicans* studied (MSH #005), 10 additional typical *C. albicans* strains were also investigated. One per cent bile salts completely neutralized the inhibitory effect of 2.0 μg/ml of gentian violet for all 10 strains, so that the described inactivation is not peculiar to the strain employed as the test organism.

Further studies were undertaken to elucidate the mechanism of inactivation of these antifungal agents by bile salts. Addition of the latter to unseeded medium in 0.5, 1 and 2% concentrations produces no change in the reaction of the medium either prior to or after 48 hours incubation at 37°C. Moreover, no difference was observed between the final reaction of the bile salts media and that of the basal medium after their inoculation with the test organism and growth at 37°C for 24 and 48 hours. Thus the effect of bile salts upon activity of antimycotic agents cannot be ascribed to any alteration of pH. Bile salts in the above concentrations also had no significant effect upon the growth curve of the test organism since growth after 24 hours and 48 hours at 37°C, measured photometrically, was essentially the same in the bile salts media as in the basal medium. Since the inactivating effect of bile salts on antifungal agents might conceivably be due to the surface tension depressing property of the former, it was decided to investigate the effect of other surface active agents. Two anionic compounds, di isopropyl napthalene sodium sulfonate (Naccosol A) and di hexyl sodium sulfosuccinate (Aerosol MA), and 2 non-ionic compounds, alkyl aryl polyether alcohol (Triton X-100) and polyoxyethylene sorbitan monooleate (Tween 80) were selected for these

studies. Cationic surface active compounds could not be tested because of their own marked growth-inhibiting activity against *C. albicans*. The minimal amount of bile salts and that of the detergents tested capable of neutralizing the inhibitory effect of a fixed amount of amphotericin B, 10 μg/ml or 25 times the MIC for C. *albicans*, was determined. It was found that whereas 0.4 mg/ml bile salts completely neutralized the growth inhibiting effect of the amphotericin B, 6.0 mg/ml of Naccosol A, 10 mg/ml of Aerosol MA, and 20% by volume of both Triton X-100 and Tween 80 all failed to do so. Higher concentrations of Naccosol A and Aerosol MA could not be tested since these concentrations were by themselves growth inhibitory for *C. albicans*. In the light of these findings it was concluded that inactivation of antimycotic agents by bile salts could not be attributed to the surface tension depressive action of the latter.

To ascertain whether bile salts exert their effect upon the microorganisms or upon the chemotherapeutic agent in the course of neutralizing the inhibitory action of antifungal compounds, the following experiments were performed: 10 ml quantities of the basal medium with and without bile salts in 0.5, 1 and 2% concentrations were seeded with a standard inoculum of *C. albicans* and incubated at 37°C for 48 hours. All tubes were then centrifuged at 2000 rpm for 15 minutes and the supernatant fluids poured off and replaced with an equivalent volume of sterile saline. The amount of each of the 4 antimycotic agents under investigation required to inhibit a similar sized inoculum of bile-exposed and unexposed *Candida* cells was then determined. No difference was observed between the concentrations of each drug required to inhibit the bile-treated and untreated cells thereby indicating that contact of *C. albicans* with bile salts for 48 hours at 37°C had not affected the susceptibility of the microorganisms. Minimal amounts of bile salts needed to neutralize the antimycotic effect of graded concentrations of gentian violet was then determined. Amounts of bile salts capable of annulling the *Candida* inhibiting activity of graded concentrations of gentian violet was directly proportional to concentration of dye employed (Fig. 1). This would indicate that the inactivating effect of bile salts is exerted upon the antimycotic drug rather than upon the organisms.

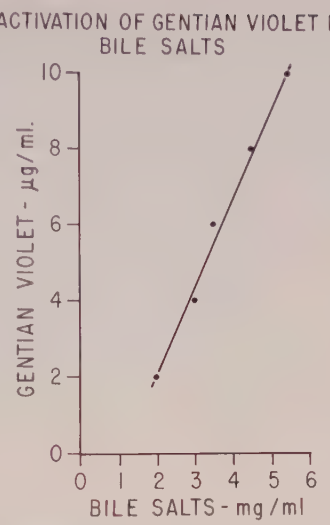

FIG. 1. Minimal amounts of bile salts capable of neutralizing *Candida* growth inhibitory effect of graded concentrations of gentian violet.

Discussion. The exact mechanism by which bile salts inactivate the different antifungal agents studied has not been established. Bile salts, in the concentrations employed, produce no pH change in the medium, exert no stimulatory or depressing effect upon growth of the test organism nor can their neutralizing effect upon antifungal agents be attributed to their property of surface tension depression. Our inability to demonstrate altered susceptibility of the microorganism plus existence of a straight line relationship between amount of bile salts needed to inactivate graded concentrations of gentian violet suggests that the former, in some undetermined manner, acts upon the chemotherapeutic agent itself rather than upon the fungus. Additional experiments to further resolve the mechanism of the reported observation as well as to determine the responsible component or components of the bile salts mixture are underway.

Each of the antimycotic drugs investigated ordinarily fails to produce significant blood levels in patients after oral administration.

This is also true of the 3 antibacterial agents previously found to be inactivated by bile salts, namely polymyxin, ristocetin and vancomycin(1). In the light of the findings herein reported, it may very well be that diminished or absent absorption of certain antifungal and antibacterial agents when administered orally is due to their inactivation by the high concentration of bile salts normally present in the intestinal tract rather than to their poor diffusion through the intestinal wall because of their large molecular size. Studies leading to reversal of bile inactivation of these antimicrobial agents could lead to their increased absorption and hence clinical usefulness.

Summary. One per cent bile salts inactivates amphotericin B, chlorquinaldol, gentian violet and nystatin to a considerable but variable degree depending upon the antimycotic agent. A number of factors possibly involved in the mechanism of this inactivation have been studied and are discussed.

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Serum Cholesterol Determinations as Affected by Vitamin A. (24309)

LOIS J. KINLEY AND R. F. KRAUSE (Introduced by E. J. Van Liere)
Department of Biochemistry, West Virginia University Medical Center, Morgantown, W. Va.

It was observed in this laboratory that Vit. A administration resulted in an increase in total serum cholesterol and that as the serum Vit. A level increased, serum total cholesterol increased proportionately. It was then necessary to determine if the elevation of serum Vit. A was associated with an apparent or real increase in total serum cholesterol.

The method used(1) for determining serum cholesterol was one widely used in clinical laboratories. Since the reagent ferric chloride is used both in this method and in measurement of Vit. A, it was felt that perhaps Vit. A was interfering with determination of cholesterol. It was therefore believed worthwhile to investigate the effect of Vit. A on the ferric chloride method of determining serum cholesterol.

Methods and materials. Serums were taken from a group of 5 patients, having the following Vit. A concentration as determined by the Carr-Price method(2); patient 1, 55; patient 2, 28; patient 3, 56, patient 4, 37, and patient 5, 40 μg% respectively. Of this group of serums, 3 had elevated cholesterol levels and 2 had normal cholesterol levels. Varying amounts of Vit. A alcohol equivalent to 15-150 μg per 100 ml serum were added to each serum (Tables I and II). In each case, a control tube was set up containing 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 ml respectively of Vit. A alcohol (3 μg per ml in Mallinckrodt chloroform) were added and the volume made up to 0.6 ml with chloroform.

A total cholesterol determination was then done on each of the 6 tubes from the 5 different serums by the method of Zak(3).

The same group of 5 serums was set up

TABLE I. Effect of Varying Concentration of Vit. A on Determination of Total Serum Cholesterol by the Zak Procedure(3).

	mg % total cholesterol						
	Control	15 μ %*	30 μ %*	60 μ %*	90 μ %*	120 μ %*	150 μ %*
Patient 1	403	417	425	455	480	510	545
2	363	363	380	415	445	470	500
3	321	326	330	360	390	420	445
4	249	253	260	295	325	360	390
5	205	208	210	230	255	280	300

* Vit. A added.

TABLE II. Effect of Varying Concentration of Vit. A on Determination of Total Serum Cholesterol by the Schoenheimer-Sperry Procedure(4).

	mg % total cholesterol						
	Control	15 μ %*	30 μ %*	60 μ %*	90 μ %*	120 μ %*	150 μ %*
Patient 1	411	412	414	415	417	420	420
2	367	366	366	363	369	370	372
3	319	320	323	325	330	328	330
4	250	253	252	254	255	255	256
5	201	202	201	203	204	205	207

* Vit. A added.

as before, and a total cholesterol determination made on each by the Schoenheimer-Sperry saponification-extraction method(4) with a slight modification.*

Results. The results, in mg% total cholesterol, shown in Tables I and II represent an average of tests run in triplicate.

With addition of 30 μg of Vit. A per 100 ml serum, and using the Zak method, there was an increase of 10-15 mg% total cholesterol. However, with the addition of greater amounts of Vit. A, 120-150 μg per 100 ml, there was an increase of 100-200 mg% total cholesterol.

The results with the Schoenheimer-Sperry saponification-extraction method, showed no appreciable elevation of total serum cholesterol, even with addition of 150 μg Vit. A per 100 ml serum. This was probably due to the fact that the Vit. A had been separated from the total cholesterol by precipitation of the latter with digitonin.

It is of interest that the control samples, with no Vit. A added, show very similar values when determined by either the Zak or Schoenheimer-Sperry methods.

Discussion. The results of this investigation demonstrate the fact that excessively high serum Vit. A levels will cause interference with cholesterol determination when the Zak procedure is used. Such interference is not noted when Vit. A is separated from the serum before cholesterol determinations are made. This observation may account for the elevations in serum cholesterols following Vit. A therapy as reported by Lasch(5).

It should also be pointed out that care must be taken by the clinical laboratory in choosing a method for determination of total serum cholesterol when high serum levels of Vit. A are suspected.

Summary. 1. A study was made of the effects of various levels of Vit. A in the serum on total cholesterol as determined by a method using a ferric chloride color reagent and a saponification-extraction method. 2. Comparison between the 2 methods showed that the Vit. A must be separated from the serum before making total cholesterol determinations by the ferric chloride color reagent method.

* The precipitate was dissolved in glacial acetic acid and ferric chloride color reagent added instead of the Libermann-Burchard reaction.

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Oxygen Consumption of Post-Mortem Human Heart Muscle.*† (24310)

WILLIAM C. ULLRICK,‡ E. A. LENTINI,§‖ AND SHELDON C. SOMMERS
(Introduced by Earl R. Loew)
Depts. of Physiology and Pathology, Boston University School of Medicine, Boston, Mass.

Excitability and contractility characteristics of post-mortem human heart muscle were previously reported by the present authors(1). This report is concerned with oxygen consumption of post-mortem human heart muscle.

Materials and methods. Human hearts were obtained from 1.8 to 8.4 hours after death. Two trabecular muscles of diameters less than 1 mm were removed from each left ventricle. One muscle preparation was positioned in clamps, placed in a constant temperature 37.5° bath and bathed continuously with oxygenated Ringer's solution. The muscle was kept under a constant resting tension of 1 g, and after a 30 minute to one hour equilibration period was stimulated at a frequency of 1/sec with 6 msec square wave pulses. Isometric developed tension was recorded. Details of the method of stimulation and recording, and composition of Ringer's solution used were as previously reported(2). The other muscle preparation from each heart was suspended in a modified Warburg flask in a vertical position. A freely hanging 1 g weight was attached to the lower end of the preparation. The muscle was not stimulated, and was under a constant resting tension of one g. The Warburg flask contained 6.9 ml of Ringer's solution and 0.1 ml of 20% KOH in a side well. Oxygen consumption, under a 100% O_2 gas phase, was determined by standard manometric technics, and is expressed as μl O_2 uptake/hr/mg dry weight of tissue (QO_2). Oxygen consumptions are reported for only those preparations, 7 in number, whose mates from the same heart responded by contraction to electrical stimulation in the muscle bath. (5 preparations, obtained within the same time interval after death, did not respond).

Results. Table I lists the primary clinical diagnosis of the patient from whom each heart was obtained, interval from death of patient to placement of the muscle preparations in bath and Warburg systems, and QO_2's and isometric tension development of the preparations from each heart. The mean QO_2 was 21.0 $\pm$ 2.5 (S.E.) and mean isometric tension development was 287 $\pm$ 137 mg. Tension is expressed simply in mg per preparation, since the preparations were approximately all the same size, (length: 5 mm; diameter: 0.7 mm; and weight: 0.6 mg, dry). There was no correlation between oxygen consumption of one preparation from an autopsied heart and capacity to develop tension by another preparation from the same heart, nor was there any correlation between oxygen consumption or tension development with the post-mortem interval.

It may be questionable whether the oxygen consumptions reported here represent respiratory rates for normal human heart tissue. The influence of the post-mortem anoxic interval on respiration is undetermined. Clinical condition of the patient at time of death may also have influenced the respiratory rate. In spite of these considerations mean oxygen consumption for post-mortem human ventricular tissue compares favorably with *in vivo* measurements for the normal human heart reported by others. For example, recently reported mean values are 21.84(3) and 22.00(4) (converting given ml O_2 utilization/min/100 g of tissue to QO_2's). Our data therefore indicate that post-mortem human heart muscle is a functionally and metabolically active tissue, and may serve as a valuable adjunct to the study

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§ During tenure of Pre-doctoral Research Fellowship of Nat. Heart Inst.

‖ Present address: Dept. of Physiology, University of Oregon Medical School, Portland.

TABLE I. Oxygen Consumption of Post-Mortem Human Heart Muscle.

Clinical diagnosis	Post-mortem interval, hr	Qo_2 Prep. A	Isometric developed tension, mg Prep. B
Hydrocephalus (still-born infant)	1.8	23	90
Rheumatic heart disease, mitral stenosis	2.0	17	1020
Generalized arteriosclerosis, involvement of coronary arteries	3.5	12	30
Arteriosclerotic heart disease	4.1	17	190
Generalized arteriosclerosis, G-I bleeding	5.0	27	150
Carcinoma of esophagus	5.5	21	55
Malignant carcinoid	8.4	31	475
Mean		21.0 ± 2.6	287 ± 137

of human cardiac physiology and pathophysiology.

Summary. Oxygen consumption measurements were made on trabeculae carneae from 7 contractile human hearts, obtained 1.8 to 8.4 hours after death. Mean QO_2 was 21.0 $\pm$ 2.5, which compares favorably with mean *in vivo* oxygen utilization values reported for the normal human heart. There was no correlation between magnitude of the oxygen uptake and isometric tension developed by a sister preparation from the same heart, nor with the post-mortem interval.

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Isolation of Partially Purified Properdin from Bovine Serum by Cold Ethanol Fractionation.* (24311)

FRED MIYA AND STANLEY MARCUS

Department of Bacteriology, University of Utah College of Medicine, Salt Lake City

Properdin, a normal serum constituent which in conjunction with complement or complement-like substances and Mg++ constitutes the properdin system, was first reported to be a natural humoral defense mechanism by Pillemer and co-workers(1). A procedure for isolation and concentration of this euglobulin substance was described. The protocol involves the adsorption of properdin to zymosan and subsequent elution under suitable conditions(2). The use of the zymosan adsorption technic generally yields final properdin solutions of variable activity since zymosan preparations vary in activity. Pillemer *et al.*(1) reported that properdin is found in serum Fraction III using the separation procedure described by Deutsch *et al.*(3). Pennell *et al.*(4) used method 6 of Cohn, *et al.*(5) and reported that 90-95% of total properdin activity of plasma was recovered in a subfraction of Fraction I, and the remaining activity appeared in Fraction III. Linder(6) has shown that the majority of properdin activity of human serum resides in the gamma globulin eluate when the serum constituents are separated electrophoretically. Experiments designed to determine the value of properdin as a therapeutic agent following exposure of animals to whole body x-irradiation made necessary the preparation of properdin solutions of high activity per unit volume in order that

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adequate concentrations of material could be administered intravenously or intraperitoneally without causing physiological stress in mice and rats. A Cohn ethanol fractionation (modified Method 6) procedure was employed in order to obtain partially purified properdin solutions without using the variable and costly zymosan. The results of a direct ethanol fractionation procedure are reported herein.

Materials and Methods. Bovine serum. The whole blood of healthy adult cows was collected in sodium dichromate-sulfuric acid cleaned glass receptacles and allowed to stand at room temperature for 2 hours. The clots were then rimmed, and the blood stored overnight at 2°C. The serum was separated by centrifugation at high speed in a refrigerated centrifuge. The clear serum was used immediately in the fractionation procedure. *Ethanol-buffer mixture.* For each liter of serum the following solution was prepared: 601 ml of 53.3% ethyl alcohol at –5°C; 0.88 ml of 10 M acetic acid at 25°C; 0.44 ml of 4 M sodium acetate at 25°C; 2.30 ml of 95% ethyl alcohol at –5°C. The above ingredients are measured out at the temperature specified and mixed together thoroughly. The entire mixture was allowed to equilibrate at –5°C before addition to the serum. *pH 7.4 Barbital buffer* was prepared according to the directions in Kabat and Mayer(7). *Constant temperature bath.* A double jacketed stainless steel container was employed. The smaller inner jacket contained a solution of sodium chloride with a freezing point depression of –5°C to –6°C. The larger outer jacket contained the freezing calcium chloride-ice mixture. *Procedure.* The glass container holding the clear bovine serum was placed into NaCl solution. The serum was stirred slowly with a mechanical agitator to hasten equilibrium conditions and prevent ice crystal formation. When temperature of the serum was 0°C, the pre-cooled (–5°C) ethanol buffer mixture was added. Rate of addition through a capillary jet was 5-6 ml per minute. Overall addition time per liter of serum was about 2 hours. The entire mixture was adequately stirred during the addition of the ethanol buffer mixture in order to prevent high ethanol concentrations near the jet tip which could cause irreversible protein changes. The final concentration of ethanol was 20% V/V. After addition of the ethanol buffer solution the mixture was continually stirred for another 60 minutes. Care was exercised to keep the serum-ethanol mixture at a temperature of -5 ± 0.5°C. The precipitate was centrifuged out at high speed in a refrigerated centrifuge (0°C). The supernate was discarded, and the centrifuge tubes were inverted 10 minutes in the refrigerator to drain the excess fluid. The precipitate was redissolved in pH 7.4 barbital buffer, (250 ml buffer/liter starting material). The redissolved protein fraction was transferred to a filtering flask, and negative pressure was applied to remove the residual alcohol. This operation was carried out at room temperature. The protein solution was cooled to 0°C for 24 hours during which time a precipitate appeared. The mixture was centrifuged and the residue collected. The supernate was discarded. The residue was treated with pH 7.4 barbital buffer (3 ml buffer per ml of residue). Suspension of the residue was obtained by gentle agitation at room temperature. The final product was stable turbid suspension and was stored at 0°C. The titers of the properdin mixtures at various steps in the isolation are shown in Table I.

TABLE I. Properdin Activity of Various Fractions Obtained during Isolation Procedure.

Material	Amt, ml	Properdin,* units/ml	mg N per ml
Bovine serum	1000	15	18.7
↓			
Redissolved ethanol precipitation fraction	250	100–200	27.5
↓			
Supernate from cold separation centrifugation	235	2–4	12.7
Residue from cold separation centrifugation	15	400	37.6
↓			
Final suspension	60	100	9.4

* Zymosan assay procedure of Pillemer *et al.* (1956).

Results. It is seen that considerable increase in activity on a volume basis was

achieved, *i.e.*, from 15 to 100 units per ml. However, the isolating procedure is carried out at a net loss, *i.e.*, from 15000 units to 6000 units or a net recovery of about 40%. The value of the procedure derives from the increased concentration per unit volume.

Anticomplementary titers of the products were run in conjunction with the properdin assays and in each case the product was shown to be non-anticomplementary. The final product had the paper electrophoretic characteristics of gamma globulin.

The purification step achieved by inadvertent discovery of the insolubility of the properdin-containing fraction at 0°C was apparently made possible by the prior concentration steps. The possible relation of "cryogenic" proteins(8,9,10) to the partially purified properdin. which acts similarly to described cryogenic globulin, remains to be determined.

Summary. A procedure for isolation of properdin from bovine serum using cold ethanol fractionation technics is reported. Results indicate that high yields of properdin per unit volume are obtainable without use of zymosan.

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Sensitized Sheep Cell Hemagglutination Reaction in Rats with Experimental Infection of Bone and Joint. (24312)

EDWIN M. LERNER II, ROBERT R. WILLIAMS AND JACK C. JENKINS

Laboratories of Pathology and Histochemistry, and of Physical Biology, N. I. H., Bethesda, Md.

A reproducible infection of bone and joint of rats has been produced in high incidence with a strain of *Streptobacillus moniliformis* isolated from a rat with middle-ear infection (1). Although this microorganism is considered of low virulence or a commensal for rats(2,3), this strain isolated in our laboratory has shown a specific affinity for joints, bone, and periarticular tissues after intravenous injection. To date, 109 out of 116 animals injected intravenously with this microorganism have developed gross and/or microscopic lesions. Of those animals showing acute changes, the infecting microorganism has been recovered from 19 of 21 wrists or ankles cultured during the acute, 5-11 day stage. Blood cultures made during this same 5-11 day stage have been positive in only 1 of 15 animals. The involvement of the region of the joint in the inflammatory process has directed immunologic studies of this infection into investigation of possible similarities to serologic reactions seen in human rheumatoid arthritis. Observations that the serum of patients with rheumatoid arthritis agglutinated sensitized bacteria, red cells, and other particles(4,5,6), have led to development of the sensitized sheep cell agglutination (SSCA) reaction as a phenomenon occurring most frequently in rheumatoid arthritis(7,8,9,10). This agglutination reaction has been found positive less frequently in some of the so-

TABLE I. Gross Changes in Rats following Intravenous Injection of *Streptobacillus moniliformis.*

Exp. No.	No. rats infected	No. rats with lesions	Regions involved				
			Left wrist	Right wrist	Left ankle	Right ankle	Other
48	8	8	3	6	3	2	
50	35	33	22	27	12	11	Left hip–1 Right hip–1
53	12	12	11	10	2	5	Right hip–1
Total:	55	53	36	43	17	18	3

called "collagen diseases," such as disseminated lupus erythematosus, polyarteritis nodosa, scleroderma, and dermatomyositis(6,8, 9,11,12,13,14). Several modifications of the agglutination reaction have been proposed and reported as yielding significantly elevated titres in 44 to 95% of patients with rheumatoid arthritis(11,12,13,14,15).

The agglutination test applied in the current experiments was a slight modification of the euglobulin test devised by Ziff *et al.*(16). Briefly, this consists of testing a euglobulin fraction of the serum proteins obtained from serum by dialysis against a low ionic strength buffer at pH approximately 6.0. This procedure enables one to use a sensitizing dose of anti-sheep erythrocyte serum as great as one-half the basic agglutinating dose without materially increasing the false positive reactions. Studies employing euglobulin fractions of serum have been reported as yielding 86-91% positive reactions in human rheumatoid arthritis(16,17,18).

Materials and methods. A. Production of lesions. Technics previously described were employed to produce lesions of joint regions in rats(1). In brief, young Holtzmann-Fisher cross rats, 2½-3½ months old, raised free of infection, were injected intravenously (tail vein) with 2.0 ml of a 20-22 hour old broth culture of *S. moniliformis.* Cultural methods have been described. A total of 55 rats in 3 separate experiments were injected, and of these, 53 developed gross changes in wrists, ankles, and occasionally elsewhere. These were manifested by redness, swelling, or tenderness of one or more joint regions, appearing usually 5 to 7 days after injection. Incidence and distribution of lesions in infected animals is shown in Table I. Animals were killed with ether on the 6th or 7th day after injection, and 2 to 5 ml of blood were drawn from the inferior vena cava. A total of 53 uninfected rats were killed with ether and bled in similar fashion to serve as normal control animals. Some of the sera from these controls were run simultaneously with sera from infected animals (Exp. 52 and 53).

B. Modified sensitized sheep cell agglutination test. 1. *Cell suspensions.* Sheep blood was collected in sterile flasks containing 100 ml of an anticoagulant and preservative solution of 0.94% disodium salt of ethylene-diamine tetraacetate (EDTA) (adjusted to pH 6.0 with NaOH) and 5.0% glucose. The cells were washed in 0.85% NaCl solution and stored as packed cells in the anticoagulant solution at 5°C. When used in tests, the packed cells were washed in saline and made to 1.5% or 3.0% suspensions of cells in a solution of one part 0.03 M EDTA (adjusted to pH 7.0), ionic strength 0.168, and 9 parts 0.85% NaCl. 2. *Sensitized cells.* Commercially available anti-sheep erythrocyte rabbit serum (Amboceptor) in 50% glycerine solution was made to 1:100 dilution of serum in 0.85% NaCl containing 0.2% phenol preservative. This was again diluted for determination of the basic agglutination titre (B.A.T.)(21), and tested against a 1.5% cell suspension. Amboceptor solution at twice the BAT dilution was added to an equal volume of 3.0% cell suspension, held at least 30 minutes at room temperature, and then overnight in the refrigerator before use. This comprised the sensitized 1.5% erythrocyte suspension. 3. *Serum euglobulin.* Sera for test were diluted with an equal volume of distilled water and were dialyzed overnight against EDTA solution (pH 6.0, ionic strength 0.0126) at 2°C to precipitate the

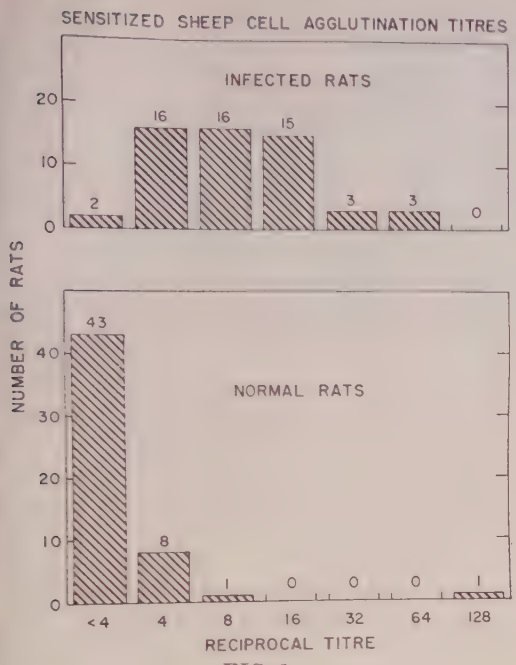

FIG. 1.

euglobulin fraction. Precipitates were washed twice in a volume of the same buffer equal to the serum volume used, and were redissolved in the pH 7 EDTA saline buffer. They were then inactivated at 56° for 30 minutes and absorbed at 37° according to Ziff(16), to remove Forsmann and other sheep cell agglutinins. 4. *Agglutination Titre.* Serial 2-fold dilutions, starting at a 1:4 dilution of rat serum euglobulin were made for the titration. 0.5 ml of 1.5% sensitized sheep erythrocyte suspension was added to 0.5 ml of each serum dilution. Tubes were held at 5°C overnight, and agglutination was read as follows. The tubes were inverted simultaneously 3 times in a covered rack, and the serum-red cell mixtures were allowed to settle for 1 hour. Agglutinations were graded from 0 to ++++, based on size and rate of settling of the red cell clumps. The highest dilution showing agglutination, *i.e.*, a definite agglutination pattern on the curved bottom of the tube, was taken as the agglutination titre(19).

Results. Serum euglobulin fractions of 55 rats infected with *S. moniliformis*, and of 53 normal uninfected rats were assayed for sensitized sheep cell agglutinins. The results of these tests and the distribution of titres developed by the 2 groups of rats is shown in Fig. 1. It will be observed that 43 of the 53 uninfected controls showed titres of less than 1:4, and 8 showed titres of 1:4. Only 2 rats developed higher titres, one of which was 1:128. In the infected group, only 2 rats showed a titre of less than 1:4, and 37 of the 55 developed titres greater than 1:8, a level which was exceeded by only 2 of the uninfected controls. This would appear to establish a titre of 1:8 as definitely abnormal, and indicative of response to this infection, under the conditions described. The possibility that the SSCA protein was not being recovered in the euglobulin fraction was excluded by testing the whole sera of uninfected rats. Of 27 such sera tested, only one had a titre of 1:4, indicating that the euglobulin method was definitely more sensitive for detection of low SSCA titres.

Discussion. An increase in sensitized sheep cell agglutinin titre was demonstrated in the euglobulin fraction of sera from rats which were experimentally infected with *S. moniliformis*. A high percentage of these infected animals developed lesions in or about joints within 5-7 days after injection of the microorganism, at which time sera were taken for testing. Although the majority of infected rats showed elevated titres, no extremely high titres were developed, and the striking difference was in the extremely low titres observed in normal controls. The absence of development of extremely high SSCA titres may be ascribed to the general lack of immunologic responsiveness noted in the rat (20), to the early acute stage of the experimental disease at which time sera were taken, or to basic differences in the human and murine diseases.

The high titres observed in human rheumatoid arthritis are not usually found until the disease has been established for 6 months or more(7,8,13,21), and only occasionally has the SSCA titre been reported elevated in early rheumatoid arthritis(22,23). Since the inflammatory process in the joint regions in these rats was at the acute stage, the finding of elevated titres in their sera is of added interest. The one significantly elevated serum titre in an animal from the uninfected group

may conceivably have been due to accidental or spontaneous infection of this animal with *S. moniliformis*, since this microorganism is extremely common in rat colonies and considerable vigilance is required to eliminate it completely.

It will be of interest to determine whether the sensitized sheep cell agglutinins of the rat are gamma globulins with a sedimentation constant of approximately 19S, as in the human serum studies of Franklin *et al.*, and whether these tend to complex with 7S gamma globulins to form complexes of 22S or greater in the same manner as in human rheumatoid arthritis. Since the current series of rats have developed low titres, and Franklin *et al.*(24) found that only high titre sera showed demonstrable complexes of 22S or greater, it may be necessary to maintain chronic or recurrent infections in order to achieve high concentrations of the high molecular weight complexes in rat sera. On the other hand, it may be necessary to investigate this phenomenon in other species.

Summary. 1) Rats infected with a strain of *Streptobacillus moniliformis* developed an inflammatory reaction in joint regions in 109 of 116 animals. 2) Sensitized sheep cell agglutination titres of the serum euglobulin tested from 55 infected rats were significantly higher than those of 53 uninfected controls.

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Observations on Mechanism and Prevention of Non-Specific Agglutination of Leukocytes.* (24313)

THOMAS E. BRITTINGHAM† (Introduced by Carl V. Moore)

Dept. of Internal Medicine, Washington University School of Medicine, St. Louis, Mo.

Leukocytes have a striking tendency to agglutinate non-specifically. Thus, leukocytes obtained by sedimentation from normal whole blood and incubated with any normal un-

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† Public Health Research Fellow of Nat. Heart Inst.

treated serum promptly and invariably agglutinate. The present study suggests that such non-specific clumping is caused by polymerization of fibrinogen adsorbed by leukocytes, and demonstrates that the clumping may be avoided either by decalcifying test sera with disodium ethylene tetraacetate or by using leukocytes obtained from defibrinated blood rather than from whole blood. A study of the mechanism and prevention of non-specific clumping of white blood cells is relevant to the construction of a satisfactory leuko-agglutinin test.

Methods. Preparation of suspension of leukocytes: 16 ml of blood were obtained from normal human donors by clean venipuncture; the blood was allowed to flow directly into a 12 × 125 mm siliconed glass tube containing 0.4 ml of a 5% solution in distilled water of disodium ethylene diamine tetraacetate (this 5% solution is hereafter referred to as EDTA) for anti-coagulation. After being inverted 10 times for mixing, the tube was tilted to a 45° angle from the horizontal, placed in the refrigerator, and the blood allowed to sediment for 2½ hours. The entire supernatant (usually 4-5 ml in volume), including the grossly visible layer of white cells immediately on top of the erythrocytes, was then removed with a capillary pipet and transferred to a 10 × 100 mm siliconed glass tube, tube B. Tube B was centrifuged at only 200 RPM for 15 minutes in a centrifuge of radius 12 cm (radius measured to top of centrifuge cup). After centrifugation, a small button of cells was present at the bottom of Tube B. After removal of the supernatant as completely as possible by capillary pipet, the residual cell button was suspended in approximately 2.5 ml of autologous (*i.e.* obtained from the donor of the particular white cells being used) serum and incubated until reading. The autologous test serum was untreated except where otherwise specified. Typical cell counts of the incubation mixture were leukocytes 18,000 per cu mm, erythrocytes 9,000 per cu mm, and platelets 120,000 per cu mm. When anticoagulants other than EDTA were used, leukocytes were obtained in an identical manner. For 16 ml of blood the following quantities of anticoagulant were used: (1) 1.6 ml 3.2% sodium citrate; (2) 1.6 ml mixed oxalate solution (2.4 g ammonium oxalate and 1.6 g potassium oxalate dissolved in 100 ml distilled water); (3) 0.12 ml heparin solution (1000 International Units per ml); (4) 1.8 ml acid-citrate-dextrose solution (formula A, USP). The procedure for obtaining leukocytes from defibrinated blood was also essentially the same, except that such blood was initially sedimented for only 2 hours, then was centrifuged for 30 minutes at 500 rpm in order to increase the initial yield of supernatant. Blood was defibrinated by taking 16 ml of blood into a glass test tube containing 6 glass beads, inverting the tube regularly for 5 minutes, then removing the resultant clot with a wooden applicator stick, leaving about 12-13 ml of defibrinated blood in the tube. The incubation mixtures were examined for agglutination by gently shaking the incubation tube to suspend the leukocytes, transferring about 0.15 ml of the leukocyte suspension to a micro test slide, depressed cell type, and reading microscopically at 100 × magnification. In an unagglutinated suspension each leukocyte lay about equidistant from its neighbor, as if there were spheres of mutual repulsion about each cell. In an agglutinated suspension, on the other hand, there were in each low power field, 15 or more tight clumps, each containing 5 or more leukocytes.

Results. Agglutination of leukocytes in normal untreated serum. Leukocytes taken from whole blood anticoagulated with EDTA were invariably clumped when suspended in autologous serum not containing an anticoagulant. Clumping was visible microscopically within 60 seconds after suspension of the leukocyte button in the serum. The results were the same whether the serum was fresh, or whether thrombin had been inactivated either by storage of the serum for several days at 4°C., or by heating to 57°C for 30 minutes. The results were also the same if isologous (from normal humans other than the leukocyte donor) sera were used (Table I). About half of the leukocytes participated in the numerous small (5-20 cells) tight clumps seen, while the other half remained unagglutinated. After several hours of incu-

TABLE I. Effect of Normal Sera and Plasmas on Normal Leukocyte Suspensions. Mixtures incubated at 37°C for one hour.

	Leukocytes obtained from whole blood anticoagulated with EDTA		Leukocytes obtained from defibrinated blood	
Test serum	Donor 1	Donor 2	Donor 1	Donor 2
Autologous				
Fresh	+	+	0	0
Aged*	+	+	0	0
Heated†	+	+	0	0
Isologous				
Fresh	+	+	0	0
Aged*	+	+	0	0
Heated†	+	+	0	0
Autologous, EDTA added‡	0	0	0	0
Isologous, EDTA added‡	0	0	0	0
Autologous plasma (EDTA)	0	0	—	—
Isologous plasma (EDTA)	0	0	—	—

* Test serum stored for 14 days at 4°C prior to use.

† Test serum heated to 57°C for 30 min. prior to use.

‡ EDTA added to concentration of 0.4 ml EDTA in 8 ml test serum.

+ = agglutination present. 0 = agglutination absent.

bation, all of the clumped cells adhered tightly together in a single mass. The agglutinated white cells seemed to include a disproportionately large number of the neutrophils present, and correspondingly a disproportionately small number of the lymphocytes. Virtually all of the platelets in the incubation suspension became involved in the leukocyte clumps, but the erythrocytes remained entirely unclumped. The sera from all 26 normal persons tested have shown this same phenomenon of agglutinating autologous leukocytes, but both acute leukemic leukocytes and the lymphocytes from chronic lymphocytic leukemic blood showed much less tendency than normal cells to agglutinate when incubated in untreated autologous serum. White cells taken from whole blood anticoagulated with agents other than EDTA, *i.e.* sodium citrate, mixed oxalate solution, heparin, or acid-citrate-dextrose solution, behaved just as did cells taken from EDTA blood in being promptly clumped by autologous serum not containing an anticoagulant.

Prevention of the agglutination of leukocytes in normal serum. If leukocytes were simply prepared from defibrinated rather than whole blood, they were never agglutinated by normal serum, even after several hours of incubation. For technical reasons, however, it may be advantageous in leuko-agglutinin testing to use leukocytes prepared from whole blood rather than from defibrinated blood. It was then found that non-specific agglutination of white cells obtained from whole blood (anticoagulated with EDTA) could be prevented by incubating the cells with EDTA plasma instead of serum, or by adding EDTA in adequate amount to the test serum. EDTA must be added to a concentration (0.4 ml EDTA in 8 ml of test serum) equivalent to that used for anticoagulating whole blood. The results of a typical experiment are summarized in Table I. White blood cells could be incubated in serum containing an adequate concentration of EDTA for more than 8 hours (at 4°C or 20°C or 37°C) without showing any agglutination whatever, although eventually clumping did occur. While non-specific clumping was suppressed by adding EDTA to the serum being tested, the action of pathologic leuko-agglutinins remained readily demonstrable and apparently unaffected by presence of EDTA.

Miscellaneous observations on EDTA action in preventing non-specific agglutination. EDTA acts by preventing agglutinates, not by dispersing them, for the agglutinates persisted if the EDTA was added to the test serum only after they had formed. If too low a concentration of EDTA (*e.g.* less than 0.2 ml EDTA in 8 ml of test serum) was used, non-specific clumping slowly occurred within the first 2 hours of incubation. Even the usually adequate concentration of EDTA in test serum did not prevent non-specific clumping of leukocytes if an excess of calcium ion was added to the serum at any time. On the other hand, if the serum had already been partially decalcified (by adding oxalate and centrifuging), a much lower concentration of EDTA than the usual adequate one sufficed

TABLE II. Effect of Different Anticoagulants in Preventing Non-Specific Leukocyte Agglutination. Cells incubated at 20°C with autologous serum.

Hr of incubation	Anticoagulant used				
	EDTA	Oxalate	Citrate	ACD	Heparin
0	0	0	0	0	+
1	0	0	+	+	+
4	0	0	+	+	+
8	0	0	+	+	+
12	0	+	+	+	+
16	+	+	+	+	+

Same anticoagulant was used in both incubation serum and in the whole blood from which leukocytes were obtained.

+ = agglutination present. 0 = agglutination absent.

to prevent false agglutination. Thus, the amount of EDTA effective in preventing non-specific clumping of white cells appears to be that amount necessary for complete decalcification of the test serum.

Relative effects of different anticoagulants in inhibiting non-specific agglutination. Leukocytes were obtained from whole blood and were incubated with autologous sera containing exactly twice the concentration of the same anticoagulant used in the whole blood from which the leukocytes were obtained. White cells from 10 normal donors were tested with each of 5 different anticoagulants. EDTA was repeatedly found to be the most effective anticoagulant in preventing non-specific agglutination (*i.e.* the longest periods of incubation elapsed before cell clumping began), but the potassium and ammonium oxalate mixture was almost as effective. Both sodium citrate and the acid-citrate-dextrose solution were distinctly less effective, some white cell agglutination usually being present after only one hour of incubation. Heparin (several different commercial preparations were tried) was by far the least useful anticoagulant, leukocytes and platelets in heparinized blood usually agglutinating within a few minutes of beginning sedimentation and long prior to beginning incubation. The results of a typical experiment are summarized in Table II. The results were unchanged when autologous plasma was used instead of serum with an added anticoagulant. Of interest was the fact that when we tried to use the mixed oxalate solution in half the usual concentration, it was, like EDTA, much less effective in preventing leukocyte agglutination than when in its usual concentration.

Mechanical trapping of cells in a fibrin clot not responsible for non-specific agglutination. It seemed possible that the agglutination of normal leukocytes by autologous serum was caused by simple mechanical trapping of the leukocytes in tiny pieces of fibrin clot formed from the small amounts of free fibrinogen inevitably present in the cell suspension when cells had been obtained from whole blood. To test this possibility, leukocyte suspensions were prepared from defibrinated blood, and to the cells during a usual incubation was added 0.02 ml of autologous plasma. The fibrinogen in the plasma was promptly clotted by the excess of calcium present. Microscopically, one saw occasional red and white cells trapped loosely in the clot. However, at no time did any leukocyte clumping occur, either inside or outside the clot. In addition, white cells were taken from whole blood and washed 2 times in saline (with added EDTA) to remove all but traces of free fibrinogen. Such cells were still clumped promptly on addition of autologous untreated serum though there was so little free fibrinogen present that no visible fibrin clot ever formed in such a preparation. Thus, mechanical trapping of leukocytes in a clot does not apparently cause the phenomenon of non-specific agglutination of white blood cells.

Discussion. The results of these experiments can be most easily interpreted by assuming that fibrinogen is adsorbed on the surface of some leukocytes, and that when polymerization of the fibrinogen occurs, as in the clotting process, cell surfaces are joined by fibrinogen-fibrinogen bonds, and "agglutination" of the leukocytes takes place. Such non-specific agglutination can be prevented by inhibiting the clotting process (*e.g.* by decalcifying test serum as well as cell preparation), or by using white blood cells which do not contain adsorbed fibrinogen, that is, by using white cells separated from defibrinated blood (see below).

More direct evidence that leukocytes do adsorb fibrinogen was provided by Seligmann, *et al.*(1), who found, using sensitive immu-

nologic technics, fibrinogen to be present in the extracts of washed leukocytes prepared from whole blood, but not in the extracts of leukocytes prepared from defibrinated blood. That normal leukocytes adsorb plasma protein whereas normal erythrocytes apparently adsorb almost none is not surprising in light of the old observation(2) that the white blood cell has a hydrophilic polar surface, in contrast to the red blood cell which has a hydrophobic non-polar surface that will not adsorb more than traces of protein in its normal uninjured state. The non-polar character of the erythrocyte surface is one important factor permitting the anti-globulin (Coombs) test to be so useful, since normal red cells give a negative test, and only an abnormal red cell surface results in a positive test. Because the normal leukocyte surface does contain adsorbed protein one would anticipate that current efforts to apply the anti-globulin test to leukocytes will not be successful.

The observation(3) that normal globulin is present in or avidly adheres to normal human leukocytes together with the observations already reported and discussed here suggest that normal leukocytes may have several kinds of adsorbed plasma proteins on their surface. Obviously, the possibility exists that a "leuko-agglutinin" may in fact be acting against adsorbed plasma protein (on a white blood cell behaving like a collodion particle) rather than against the leukocyte itself. This possibility must at present be considered always in interpretation of leuko-agglutinins.

Because of the inability to demonstrate fibrinogen attached to the surface of white cells prepared from defibrinated blood, some authors have concluded that the fibrinogen must be deposited on leukocytes only *in vitro*. However, when one defibrinates blood, a large fraction (usually about one-half) of the leukocytes originally present in the blood are inevitably removed with the clot, and the leukocytes removed might have contained adsorbed fibrinogen *in vivo* and so have been separated with the clot. Furthermore, Allison, Smith and Wood(4), on the basis of their observations of the leukocytic sticking reaction in the early stages of thermal inflammation, have suggested that partially polymerized fibrinogen may be present on leukocyte surfaces at this time. The explanation for either why all leukocytes do not adsorb fibrinogen *in vivo* or for the alternate possibility that leukocytes only adsorb fibrinogen *in vitro* is not clear.

That EDTA is highly effective in inhibiting fibrinogen polymerization and therefore non-specific clumping of leukocytes is not surprising in view of the work of Rosenfeld and Janszky(5). They found that EDTA, in addition to preventing thrombin generation, specifically inhibited fibrinogen-fibrin transformation, an effect they attributed to the potent decalcifying action of EDTA. We have supposed that the greater effectiveness in preventing non-specific leukocyte agglutination of EDTA over other decalcifying anticoagulants results because EDTA binds calcium more tightly than do the other agents. Thus, EDTA may inhibit blood clotting more completely than, for example, the usual amounts of sodium citrate. The inevitable eventual clumping of leukocytes taken from whole blood may result from the fact that fibrinogen polymerization occurs constantly, though very slowly, in any whole blood or plasma removed from the body. Also one would expect that the leukocytes themselves, which are known to contain thromboplastic substances(6) and proteolytic enzymes, might accelerate fibrinogen polymerization as they break up and release these clot-promoting substances.

The non-specific agglutinates of leukocytes may look precisely like the leukocyte clumps produced by pathologic sera. Consequently, just as in all agglutination tests, one should not infer the presence of an antibody simply because one observes clumped cells. Although there are probably other mechanisms of non-specific agglutination of white blood cells(7), the mechanism here described is a common and important one, and inhibition of this one kind of non-specific agglutination (by adding EDTA to the test sera) has enabled a satisfactory leuko-agglutinin test(8) to be constructed.

Because platelets regularly adsorb fi-

brinogen(1), it is probable that our findings are relevant to the platelet as well as to the leukocyte.

Summary and conclusions. Fibrinogen and other plasma proteins are apparently adsorbed on the hydrophilic surface of normal leukocytes, thereby complicating the interpretation of leuko-agglutinin tests. Normal leukocytes separated from whole blood (but not leukocytes separated from defibrinated blood) were rapidly and non-specifically agglutinated when suspended in normal untreated serum. Polymerization of adsorbed fibrinogen appeared to be the mechanism of such non-specific agglutination, which could be prevented by adequate decalcification of the suspending serum. Addition of EDTA to all test sera has permitted construction of a satisfactory leuko-agglutinin test using white cells separated from whole blood.

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Effect of D-Sorbitol on Absorption of Vitamin B_{12} by Pernicious Anemia Patients.* (24314)

LEON ELLENBOGEN, VICTOR HERBERT† AND WILLIAM L. WILLIAMS

Nutrition and Physiology Dept., Research Division, Amer. Cyanamid Co., Pearl River, N. Y., and Dept. of Hematology, Montefiore Hospital, N. Y. City

It has been previously demonstrated that sorbitol in solution with Vit. B_{12} will increase oral absorption of Vit. B_{12} in non-pernicious anemia subjects as measured by an increase in serum levels. Increased absorption of Vit. B_{12} by means of sorbitol has been reported to occur in experiments with young, healthy adults(1), and intact rats(2), while limited experiments with pernicious anemia patients were inconclusive(3). Since pernicious anemia is the primary state in which increased Vit. B_{12} absorption is desired, and since it was speculated that sorbitol may have an intrinsic factor-like action, experiments were undertaken on the effect of sorbitol on absorption of orally administered Vit. B_{12} in pernicious anemia patients in remission.

Method. Each pernicious anemia patient was fed an oral dose of 2 μg of Vit. $B_{12}Co^{58}$ containing 0.25 μc of radioactivity, first alone, then together with a potent intrinsic factor concentrate, then together with crystalline D-sorbitol and finally together with both intrinsic factor concentrate and D-sorbitol. In each of these 4 tests the medication was mixed and diluted to 100 ml, and following ingestion the container was rinsed with tap water and the rinsings fed to the subject. Immediately and 24 hours after each oral dose, 1000 μg of unlabeled Vit. B_{12} was given intramuscularly. The 4 excretion studies on each patient were repeated with a second series of studies using 30 μg oral doses of $B_{12}Co^{58}$ (containing 0.5 μc of radioactivity). In all instances except where noted the amount of sorbitol used was 10 g. Radioactivity of each 24-hour urine specimen was determined with a special beaker of one liter capacity adapted to fit over and around a NaI well type scintillation crystal(4) or by counting 200 ml in

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† Senior Research Fellow, N. Y. Heart Assn. Present address: Mt. Sinai Hospital, N. Y. C.

TABLE I. Effect of Sorbitol on Absorption of Vitamin B_{12} in Pernicious Anemia Patients.
(2 μg oral dose.)

Pt.	B_{12}	B_{12} + sorbitol*	B_{12} + IF	B_{12} + IF & sorbitol*
	Excretion in urine (48 hr), μg			
K.M.	.02	.02	.36	.29
A.S.	.01	.01	.17	.23
R.U.	.01	.01	.35	.31
F.W.	.02	.02 .02 (1 g) .02 (.1 g)	.14	.11
H.W.	.01	.01	.11	.17
Avg	.02	.02	.23	.22

* Dose of sorbitol was 10 g, otherwise given in parentheses.

TABLE II. Effect of Sorbitol on Absorption of Vitamin B_{12} in Pernicious Anemia Patients.
(30 μg oral dose.)

Pt.	B_{12}	B_{12} + sorbitol*	B_{12} + IF	B_{12} + IF & sorbitol*
	Excretion in urine (48 hr), μg			
K.M.	.24	.09	.18	.18
A.S.	.44	.14 (21 g)	.45	.23 (21 g)
R.U.	.48	.03	.60	.48
F.W.	.16	.20	.24	.42
H.W.	.12	.12	.27	.36
Avg	.29	.12	.35	.33

* Dose of sorbitol was 10 g, otherwise given in parentheses.

a highly efficient plastic scintillator slightly larger than that previously described(5). The intrinsic factor concentrates used in these studies were either #186-1 (50 mg) previously studied by Baker and Mollin(6), Callender and Evans(7), Williams *et al.*(8) and Ellenbogen *et al.*(9) or preparation #1-R (30 mg). Each dose was administered to the patients by one individual and radioactivity of all the urine specimens of all the patients was determined by one individual.

Results. The results of the urinary excretion tests using both 2 μg and 30 μg oral doses of B_{12} are presented in Tables I and II respectively. Each of the 5 patients excreted 0.02 μg or less when given 2 μg $B_{12}Co^{58}$ alone indicating that the patients lacked ability to absorb Vit. B_{12}. None of the patients showed an increase in excretion of Vit. B_{12} when 10 g of sorbitol was given together with 2 μg of Vit. B_{12}. In one patient (F.W.) 1.0 and 0.1 g of sorbitol was also given to investigate the possibility that the 10 g dose might be too large and result in inhibition. The lower doses of sorbitol also did not cause an increase in absorption of Vit. B_{12}. On the other hand, when intrinsic factor concentrate was fed together with B_{12}, absorption of Vit. B_{12} was increased as indicated by increase in its urinary excretion. Excretion of B_{12} with intrinsic factor concentrate was 5 to 18 times that obtained with 2 μg alone or with 2 μg and sorbitol. When Vit. B_{12} was given simultaneously with intrinsic factor concentrate and sorbitol no increase in absorption was observed above what was obtained with Vit. B_{12} plus intrinsic factor concentrate. In 2 of the patients a slight increase of questionable significance was observed. The overall average excretion of the 5 patients receiving Vit. B_{12} plus intrinsic factor concentrate and sorbitol was 0.22 μg as compared to 0.23 μg obtained with Vit. B_{12} and intrinsic factor.

In the tests with the 30 μg oral dose of Vit. B_{12}, sorbitol showed no effect in increasing absorption obtained with 30 μg alone. On the contrary, sorbitol caused a marked inhibition of absorption in 3 of the 5 patients. Simultaneous administration of sorbitol and intrinsic factor concentrate together with Vit. B_{12} resulted in an average excretion of 0.33 μg as compared to 0.35 μg with intrinsic factor concentrate and Vit. B_{12} alone, indicating that sorbitol did not appear to enhance the effect of intrinsic factor with large oral doses of Vit. B_{12} in pernicious anemia patients.

These studies clearly indicated that sorbitol did not enhance absorption of Vit. B_{12} in pernicious anemia patients. Intrinsic factor is the only substance known to increase absorption of physiological amounts of Vit. B_{12} in pernicious anemia patients.

Summary. 1) In the urinary excretion test 5 pernicious anemia patients excreted radioactivity corresponding to an average of 0.02 μg of Vit. B_{12} when fed 2 μg of Vit. B_{12} orally and excreted an average of 0.02 μg when fed 2 μg of Vit. B_{12} together with 0.1 to 10 g of sorbitol. These patients excreted an average of 0.23 μg when fed 2 μg of Vit. B_{12} with intrinsic factor and an average of 0.22 μg when 2 μg of Vit. B_{12} were fed together with intrin-

sic factor and 10 g of sorbitol. 2) These 5 anemia patients excreted an average of 0.29 μg when fed 30 μg of Vit. B_{12} and 0.12 μg when fed 30 μg of Vit. B_{12} together with 10 to 21 g of sorbitol. The same 5 patients excreted an average of 0.35 μg when fed 30 μg of Vit. B_{12} with intrinsic factor, and excreted 0.33 μg when 10 to 21 g of sorbitol was fed together with 30 μg of Vit. B_{12} and intrinsic factor. 3) Sorbitol had no effect in promoting absorption of Vit. B_{12} in pernicious anemia patients.

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Clinical and Laboratory Studies of Mumps II. Detection and Duration of Excretion of Virus in Urine. (24315)

JOHN P. UTZ, CLARENCE F. SZWED, AND JULIUS A. KASEL
National Institute of Allergy and Infectious Diseases, N. I. H., Bethesda, Md.

The cultivation of mumps virus in tissue culture was first reported by Weller and Enders(1). Tissue cultures were prepared by suspension of amniotic membranes from embryonated hen's eggs in a manner similar to that previously described by Maitland and Maitland(2). Subsequently Kilham and Murphy(3) propagated mumps virus in mouse embryo tissue culture employing a method developed by Earle and associates. Henle and others(4) using mono-layer cell culture demonstrated cytopathic effects produced by mumps virus. Henle and Deinhardt(5) later described the use of the same method in the primary isolation of virus. We(6) reported studies on cytopathic effects of virus in tissue culture and described methods for use by the diagnostic virology laboratory in isolation of virus from a variety of patient specimens. The presence of virus in urine specimens was demonstrated in 6 patients. In the preparation of these specimens it was found necessary to dilute urine 10-fold in saline to avoid a non-specific "toxic" effect on the tissue culture cells. We felt, however, that this dilution could reduce the chances of detecting virus in lightly infected urine specimens. We here describe an improved method for preparation of urine for viral isolation and report on duration of excretion of virus in 13 additional patients.

Patients. A study was made of 11 male adults, 1 male child and 1 female child, each of whom had an illness clinically characteristic of mumps. With the exception of the 2 children, all patients were hospitalized at the Clinical Center of the Nat. Inst. of Health or at the Nat. Naval Medical Center, Bethesda, Md. Clinical diagnosis was made on physical examination at the time the patient was placed on the study. Date of onset of parotid or submaxillary gland swelling or tenderness was recorded. Follow-up examinations were made of the salivary glands at the time subsequent specimens were collected from hospitalized patients. Days of illness were numbered in relation to the first day on which the patient observed salivary gland swelling or pain. In

TABLE I. Results of Urine Culture for Mumps Virus.

Patient	Day of illness (after salivary gland swelling or pain)																		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	
R.W.				+					+		+									
R.C.				+		0		0			0									
G.D.		+			+		+					+		+		0				
W.K.				+			+		+					0		0		0		
D.M.				+				0			0		0							
R.S.			0		+					0		0		0			0			
G.S.				0	0			0						+						
C.M.		0		0		+		0		0			0		0		0		0	
J.G.			+		+			+		+		+			0					
H.O.				+			+		+		0					0				
R.O.						+					0		0					0		
D.K.			+		+			+		0		0				0			0	
P.P.	+																			
Total (+)	1	1	2	5	4	2	3	2	3	1	1	2	0	2	0	0	0	0	0	29
Total tested	1	2	3	7	5	3	3	6	3	4	5	4	3	4	2	4	2	2	2	65
% (+)	100	50	66	71	80	66	100	33	100	25	20	50	0	50	0	0	0	0	0	45

0 = tested, and negative. Blank = not tested.

none of these patients were other prodromal symptoms noted earlier than 24 hours prior to salivary gland involvement. Clinically detectable orchitis was not present in any of the patients.

Methods. The first voided morning urine specimen was collected in a sterile container. Saliva specimens collected concurrently were handled as previously described(6). Within 6 hours of collection, urine specimens were prepared by 2 methods for inoculation. *Preparation by dilution*: 0.2 ml of the urine specimen was diluted 10-fold in Hanks' balanced salt solution. *Preparation by concentration*: Approximately 15 ml of urine was clarified by centrifugation at 4°C for 10 minutes at 1500 rpm. 11.4 ml of the supernatant urine was then centrifuged at 4°C for 90 minutes at 40,000 rpm in a Spinco Ultracentrifuge, Model L. The pellet from this was then resuspended in 1.14 ml of Hanks' balanced salt solution. 0.1 ml amounts of both preparations were inoculated into HeLa and primary monkey kidney roller tube cultures. Mumps virus was identified in tissue cultures exhibiting characteristic cytopathic effects by hemagglutination inhibition tests using standard mumps antiserum(6).

Results. A total of 65 urine specimens from 13 patients were collected during the period following onset of parotid swelling. Virus was isolated from the urine as early as the first day of swelling and as late as the fourteenth day. The overall experience in isolation of virus as correlated with day of illness is shown in Table I. Forty-five percent of all specimens tested within 19 days after onset yielded virus. During the first 14 days of illness 55% of the specimens were positive and during the first 9 days of illness 70% of the specimens were positive. Virus was recovered from each of these 13 patients. Fifty-seven of the 65 specimens were prepared by both dilution and concentration methods. 25 of the 57 yielded virus: in 8 both preparations, and in 17 the concentration preparation only, were positive. In the remaining 8 of 65 specimens studied, only the dilution preparation was made: of these, 4 were positive.

The relative efficiency of monkey kidney and HeLa cell tissue culture in primary isolation of mumps virus from urine was compared. 27 of 29 isolations were made in monkey kidney cells only and the remaining 2 isolations were made in both monkey kidney and HeLa cultures. In no instance was virus isolated in HeLa cultures alone.

In 7 of 13 patients virus was detected in the urine after clinically evident parotitis had subsided whereas in 5 patients, urine specimens were negative after swelling had disappeared. Urine specimens from the 13th patient were not tested after the first day of illness.

On 63 occasions saliva specimens were collected when urines were obtained. A large number of the saliva specimens, however, were contaminated by a fungus and tissue culture studies for virus on these specimens were unsatisfactory. On 13 occasions, however, virus was detected in urine specimens but not in satisfactory saliva specimens collected at the same time. In this and the previous study (6) virus has not been recovered from saliva or swabs of the oral cavity at a time later than the 8th day of illness. Virus has been recovered from the urine of 8 patients at times later than this in these studies.

Discussion. The success of concentration of infected tissue culture fluids by high speed centrifugation for demonstration of hemagglutinin(6) suggested the use of this technic for preparation of urine specimens prior to inoculation. The superiority of this technic over the previously used method of preparation seems evident from the data presented here. This concentration procedure results in a 100-fold greater inoculum of the urine specimen that is presumably virus. Monkey kidney cell cultures seem preferable to HeLa cells for detection of mumps virus. This confirms previous work(6) but is not at odds with another observation that mumps virus once adapted to tissue culture propagates well and produces a cytopathic effect in HeLa cells. The concentration technic would seem to be adaptable to studies of other viruses and its use in isolation of another virus is being reported(7). Use of the concentration preparation and monkey kidney cell cultures inoculation results in a relatively efficient method for isolation of mumps virus during the early stages of illness. It is possible that blind serial passage of the negative tissue cultures (unpublished) and use of the hemadsorption test(8) may increase the percentage of urines that are positive.

It is of interest that virus was isolated from urine as early as the first day of illness and at a time less than 20 hours after the appearance of the first symptom. This is consistent with the concept that at the onset of parotid swelling widespread dissemination of virus has occurred. Furthermore, excretion of virus seems to continue during the phase of acute clinical illness. The mechanism by which virus is excreted in urine has been speculated upon elsewhere(7). The role that virus present in urine plays in the clinical illness is under investigation. In addition, viruria persisted in some patients for a period of time after all symptoms or signs of illness had disappeared. The small number of patients in this study and relatively short periods of observation (less than 20 days) did not make it possible to determine whether some mumps patients might excrete the virus for much longer periods. The possibility of this occurrence has epidemiologic implications as to where virus may be harbored between epidemics.

Summary. 1. Specimens of urine from mumps patients were concentrated by ultracentrifugation prior to tissue culture inoculation. This concentration resulted in 3 times as many mumps virus isolations as simultaneously tested portions of the same urine prepared by simple dilution. 2. All isolations were made in primary monkey kidney cell cultures; only 2 were made also in simultaneously inoculated HeLa cultures and none were made in HeLa cultures only. 3. Mumps virus was recovered from the urine of each of 13 patients within the first 14 days after onset of salivary gland pain or swelling. Virus was isolated from urine as early as the first and as late as the 14th day of illness. No testing was done beyond the 20th day.

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Acceleration of Vitamin E Deficiency by Torula Yeast. II. Effect of Torula Yeast Ash and Lipid. (24316)

JOHN G. BIERI, GEORGE M. BRIGGS AND CLIFFORD J. POLLARD
Laboratory of Nutrition and Endocrinology, Nat. Inst. of Arthritis and Metabolic Diseases, P. H. S., Bethesda, Md.

In previous publications from this laboratory it was shown that addition of 7.5-30% of *Torula* yeast to purified diets which were free of vit. E and selenium caused an increase in incidence and in rate of development of exudative diathesis in chicks(1,2). Part of the anti-vit. E property of *Torula* yeast was found to be due to the inorganic fraction, whereas no effect was apparent with the lipid from the yeast. Subsequently, Dam *et al.*(3) reported that samples of *Torula* yeast contained considerably more lipid than we had found, and they postulated that the unsaturated nature of the lipid could account for the anti-vit. E effect of the yeast. It is here shown that both the inorganic and lipid fractions of *Torula* yeast have anti-vit. E effects, and further, that a combination of the lipid and the ash of *Torula* yeast will duplicate the exudate-promoting properties of the whole yeast. The relatively high unsaturated fat content of the yeast reported by Dam *et al.* (3) is confirmed.

Methods. Feed grade *Torula* yeast* was used in all studies. Ashes of the yeast were prepared by incineration in a muffle furnace at 250°C for 2-3 hours, then at 550°C overnight. Average yield was 7%. When a fixative was used, magnesium carbonate equal to 8% of the weight of yeast was first mixed with the yeast. For isolation of the lipid from *Torula* yeast, 1200 g of yeast were mixed with 1200 ml of 95% ethanol and 6 l of 6N HCl. After refluxing for 1½ hours the mixture was cooled, 3 l of ethanol added, and extracted in batches with a 1:1 mixture of benzene-petroleum ether. The solvent was evaporated *in vacuo* and the dark brown oil dissolved in ethanol to facilitate mixing in the diets. To characterize the lipid fractions of yeast, 25 g samples of yeast were extracted with chloroform in a soxhlet apparatus. The material obtained was labelled "free lipid". The extracted yeast was then refluxed for 2 hours with 5 N NaOH,† cooled, neutralized with HCl and extracted successively with petroleum ether, diethyl ether and benzene. The combined extracts were washed with water, dried over Na_2SO_4 and evaporated to dryness *in vacuo*. The residue was dissolved in hexane, filtered and the solvent removed. This material was called "bound lipid." The fatty acids from the free and bound lipids were obtained after saponification and analyzed for polyunsaturation by a modification of the alkali conjugation method of Wiese and Hansen(4). In the nutritional studies, day old New Hampshire chicks, 8 per group, were fed diet C47A(2) but with an additional 0.2% DL-methionine. This diet, designated C47B, contained in %: soybean protein‡ 28, cerelose 61, vit. E-free lard§ 4, salts A 6, DL-methionine 0.8, and vitamin mix 0.2. Additions to this diet, were at the expense of cerelose. The chicks were housed in electrically heated batteries and permitted food and water *ad libitum*. After the 14th day, they were inspected daily for symptoms of exudative diathesis. Experimental periods were 28 days. In evaluating the symptoms, in addition to the incidence, average time of appearance of exudates was also calculated. Compared with the group receiving *Torula* yeast, these 2 factors indicated the exudate-promoting effect of the test substance. However, due to variation in initial stores of tocopherol by individual chicks, over-all incidence has more significance than time for appearance of symptoms.

Results. Since our previous studies(2,5) had shown that certain ashes of *Torula* yeast

* Feed grade 1N, Lake States Yeast Corp.

† Dam, *et al.*(3) obtained more lipid from alkaline than acid hydrolysis.

‡ Drackett assay protein C-1.

§ Stripped lard, Distillation Products Industries.

TABLE I. Effect of *Torula* Yeast Ash and Lipid in Promoting Exudative Diathesis.

Additions to diet C47B	Avg wt, 4 wk (g)	No. of cases*	Avg No. of days†
Exp. 1			
None	305	3/8	23.3
15% *Torula* yeast (TY)	294	6/8	16.8
1.05% TY ash‡	307	5/8	24.0
2.2% TY $MgCO_3$ ash‡	324	7/8	21.3
Exp. 2			
None	265	3/8	25.7
15% *Torula* yeast (TY)	266	6/6	18.0
1% TY lipid§	298	6/7	21.2
1% TY ash‡	310	7/8	21.6
1.8% TY $MgCO_3$ ash‡	273	6/8	22.0
1% TY lipid§ + 1.8% TY $MgCO_3$ ash‡	293	7/8	18.0

* No. of chicks with exudative diathesis/No. of chicks in the group.

† Avg No. of days in which symptoms first appeared.

‡ Prepared at 550°C. Amt of ash added was equivalent to that from 15% of yeast.

§ Equivalent to the lipid from 15% of yeast.

promoted exudates, further investigation was carried out on the possible inorganic component or components in the yeast which were responsible. Based on the mineral content of *Torula* yeast as determined from spectrographic analyses of a wet ash and from analytical data in the literature, numerous elements, as their salts, were added to the basal diet at one or more levels. Elements tested individually, and their dietary concentration in ppm were: Al, 1 and 20; As, 10, 50 and 100; Cd, 10; Mo, 10; Sb, 10; Tl, 10; Sr, 5; Si, 20 and 40; Zn, 25 and 50. A trace element mixture tested at both 5 and 10 ppm for each element contained: Al, B, Br, As, Cr, Co, F, Mo, Ni, Sr, and V. None of these additions had any effect on development of exudative diathesis.

Ashes prepared with magnesium carbonate as a fixative for possible volatile elements appeared to accelerate exudates slightly faster than did ashes without the fixative. A typical result is reported in Table I, Exp. 1. Subsequent testing, however, revealed that when magnesium oxide was added to the diet, together with the plain ash, the effect was similar to that of the ash prepared with magnesium carbonate. Addition of magnesium oxide alone to the diet, in the amount present in the ashes, did not cause formation of exudates.

To determine whether the observed effect of the ash from *Torula* yeast was restricted to this strain of yeast, the ash of brewer's yeast was also tested. In one trial, at a dietary level of 1.8%, equivalent to 30% of yeast, the ash did not produce exudates. In a second trial 1.2% of the ash, equivalent to 20% of brewer's yeast, produced exudates in 7 of 8 chicks compared with 3 of 8 on the basal diet and 7 of 7 receiving 20% of *Torula* yeast. It is known that brewer's yeast contains biologically active selenium in the form of Factor 3(6,7), and it may be that some variation in ashing conditions resulted in incomplete volatilization of the selenium in the ash used in the first trial.

In attempts to simulate the effect of *Torula* yeast ash,‖ salts of the major constituents of the ash were tested at dietary levels corresponding to 10-30% of the yeast. Compounds tested individually were 0.12 and 0.44% $CaSO_3 \cdot 2H_2O$, 0.35 and 1.3% KH_2PO_4, 1.5 and 3.0% $K_2S_2O_5$ and 1.65% $Ca_3(PO_4)_2$. None of these salts nor a 2% increase in the dietary salts A, promoted formation of exudates. A "synthetic" ash of *Torula* yeast was prepared by mixing 22.2 g of $CaSO_3 \cdot 2H_2O$, 74.0 g of KH_2PO_4 and 10.9 g of $MgCl_2 \cdot 6H_2O$ and heating at 550°C for 24 hours. The results with this material will be reported below.

When Dam *et al.*(3) reported that *Torula* yeast from the same source* as ours contained more lipid than we had previously found, it was decided to reinvestigate the effect of the lipid. The amount and fatty acid composition of the lipid obtained as described above from *Torula* yeast, as well as that from brewer's yeast, is given in Table II. *Torula* yeast contained about 6% total lipid, half of which was in the bound form, while brewer's yeast contained only 3% total lipid. Fatty acids account for one-half of the lipid in *Torula* yeast, and for about one-third of the brewer's yeast lipid. The most marked difference between the lipids from the 2 yeasts is in the content of polyunsaturated fatty acids. 45%

‖ Major cations in feed grade *Torula* yeast are: Ca 0.57, P 1.68, K 1.88, and Mg 0.13% (N.R.C. Comm. on Feed Composition, Publ. 449, 1956). Sulfur content averages about 1.2%(8).

TABLE II. Composition of Lipids Isolated from *Torula* Yeast and Dried Brewer's Yeast.*

	Torula	Brewer's
	% of whole yeast	
Free† lipid	3.01	2.03
Bound† "	2.93	.98
Total "	5.94	3.01
Free fatty acids	1.41	.75
Bound " "	1.68	.42
Total " "	3.09	1.17
	% of total fatty acids	
Unsaturated fatty acids:		
Diene	45.4	3.8
Triene	4.5	.0
Hexaene	4.7	3.5

* Pabst grade R2F.
† See text for interpretation of these terms.

of the fatty acids from *Torula* yeast are dienoic acid whereas only 3.8% of the acids from brewer's yeast contain 2 double bonds. These values agree with the analyses of Dam *et al.*(2), although the amount of *Torula* yeast total fatty acids they found was somewhat higher. Small amounts of trienoic and hexaenoic acids were present in the *Torula* yeast, but there was no significant amount of tetraenoic or pentaenoic acids.

The results of an experiment in which *Torula* yeast lipid was fed alone or with the ash are given in Table I, Exp. 2. Whereas all of the treatments increased the incidence of exudative diathesis over that in the basal group, only the combination of lipid plus $MgCO_3$ ash produced the onset of symptoms as quickly as did the intact *Torula* yeast. Since isolation of large quantities of *Torula* yeast lipid was technically impossible, and inasmuch as the lipid by analysis contained almost 50% dienoic acid, it was decided to use an oil of similar composition for further studies.

An oil which contains this amount of linoleic acid, and which also is very low in vit. E, is fractionated tall oil.¶ According to the manufacturer, this oil contains 96.8% free fatty acids, of which 48.0% is linoleic and 50.0% is oleic acid. Addition of 1% of this oil to a diet would provide 0.48% of dienoic acid. Using the value of Dam *et al.*(2) for total fatty acid content of *Torula* yeast, and the total polyunsaturated fatty acid value in Table I, 15% of *Torula* yeast in a diet would provide approximately 0.37% of these fatty acids.

¶ Acintol FA-2, Arizona Chemical Co., N. Y.

The results of feeding 1% tall oil, with or without the ash of *Torula* yeast, are given in Table III. 15% of *Torula* yeast produced the usual high incidence of exudative diathesis (90%). A similar high incidence (86.5%) was produced by a combination of tall oil and synthetic ash; tall oil plus *Torula* yeast ash gave a slightly lower incidence, 80.5%. The next highest incidence was produced by *Torula* yeast ash alone (72.4%), followed by tall oil alone (57.0%). The synthetic ash (described above) by itself resulted in only 38.4% of the birds having exudates. Except for the group receiving the synthetic ash, all groups had a similar average time for appearance of symptoms.

It is apparent that the exudate-promoting action of *Torula* yeast can be duplicated by a combination of unsaturated fatty acids and minerals similar to those present in the yeast. Not only did this combination produce a high incidence of symptoms, but in one experiment it also produced a severity of hemorrhagic exudates similar only to those found in the chicks fed the diet containing *Torula* yeast. From these results, it can be concluded that the anti-vit. E property of *Torula* yeast is due predominantly to its content of (1) polyunsaturated fatty acid and (2) min-

TABLE III. Incidence of Exudative Diathesis Produced by Tall Oil and *Torula* Yeast Ash.*

Addition to diet C47B	Avg wt, 4 wk (g)	No. of cases	%	Avg No. of days
None	264	7/30	23.3	22.6
15% *Torula* yeast (TY)	312	27/30	90.0	19.8
1% TY ash†	292	21/29	72.4	21.9
1% tall oil	275	16/28	57.0	20.4
1% TY ash + 1% tall oil	314	25/31	80.5	20.6
1% synthetic TY ash‡	269	5/13	38.4	24.2
1% synthetic TY ash + 1% tall oil	304	13/15	86.5	21.9

* Data are avg of 4 separate experiments, except the last 2 groups which are from 2 experiments.
† Prepared at 550°C. Equivalent to 15% of *Torula* yeast.
‡ See text for composition.

erals (ash). With respect to the latter, our studies suggest that it is a combination of elements rather than any single element which contributes to the pro-oxidant property of the yeast.

In other experiments, it was found that 1% of the ash of dried brewer's yeast when fed with 1% of tall oil produced a high incidence of exudative diathesis similar to that of *Torula* yeast ash plus tall oil. On the other hand, feeding an additional 2% of the salt mixture used in the C47B diet, together with 1% of tall oil, gave a low incidence of exudates. Thus, it appears that the combination of elements in *Torula* yeast, and possibly also that in brewer's yeast, has a remarkable pro-oxidative property. It is unlikely that any constituents of *Torula* yeast, other than the lipid and ash, contribute significantly to its anti-vit. E effect.

Summary. When added to a purified basal diet low in vit. E and selenium, the ash of *Torula* yeast was found to promote exudative diathesis. This effect could not be demonstrated by any of the minor elements, nor by any of the major components, of the ash. The lipid isolated from *Torula* yeast also promoted exudates; this effect could be duplicated by tall oil. A combination of lipid and ash from *Torula* yeast produced an incidence of exudative diathesis similar to that produced by an equivalent amount of the intact yeast. A combination of tall oil and a synthetic ash was just as effective. It is concluded that the anti-vit. E property of *Torula* yeast is due essentially to both its unsaturated fatty acid and mineral contents. The relatively high content of dienoic acid in the lipid of *Torula* yeast was confirmed.

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Lactic Dehydrogenase Activity in Human Semen.* (24317)

JOHN MACLEOD AND FELIX WRÓBLEWSKI

Depts. of Anatomy, Cornell University Medical College, and of Medicine and Division of Enzyme and Metabolic Studies, Memorial Center for Cancer and Allied Diseases, N. Y. City

The lactic dehydrogenase (LD) is present in normal human blood plasma and in increased concentration in myelogenous leukemia(1). The enzyme is present also in serous effusions and cerebrospinal fluid and in elevated amounts when these fluids contain or bathe growing malignant cells(2). Similarly, the proliferation of neoplastic human cells in tissue culture is accompanied by increasing lactic dehydrogenase (LD) activity of the bathing medium(2). The rapid cellular proliferation which is characteristic of human spermatogenesis, the high zinc content of human semen(3) and the presence of zinc in the molecule of LD, suggested LD activity might be present in semen. Accordingly, this possibility was investigated in the normal ejaculate and in semen derived from infertile and sterile individuals.

Methods. Lactic dehydrogenase activity of semen was estimated using 0.025 ml of a 1:10 dilution of human semen. The aliquot of semen was added to 0.6 ml 0.1 molar phosphate buffer, pH 7.4, and 0.05 ml reduced diphos-

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phopyridine nucleotide (2 mg per ml). After an incubation period of 20 minutes, 0.1 ml of sodium pyruvate (0.5 mg per ml) was added and the change in optical density was measured at 340 mμ using a 0.8 ml capacity cell with a 1 cm light path. One unit of semen LD activity is defined as that which results in a change in optical density of 0.001 per minute per 1.0 ml semen at 26°C under specific conditions. Normal semen specimens were obtained from young healthy adults and specimens of varying degrees of potential fertility from individuals attending the out-patient clinics of The New York Hospital. Estimation of sperm count, motility and morphology were done by standard methods(4). Lactic dehydrogenase activity was measured in whole semen, the centrifuged supernatant cell-free fluid and in the "split" ejaculate. The "split" ejaculate describes the semen derived when the ejaculate is partitioned into 2 or more aliquots at the time of ejaculation. This method has been used to determine the contributions of the prostate and ampulla of the ductus deferens, and of the seminal vesicles to the cellular and fluid constituents of semen(5).

Results. LD activity of the whole semen of 65 individuals ranged from 1600 to 18,000 units with a mean activity of 5600 ± 470 units. When these values are compared with those obtained in normal human blood plasma (range 200-500 units) it is obvious that the LD activity of human semen is very high. In 10 instances, the spermatozoa were separated from the seminal fluid by centrifugation. Mean LD activity of the supernatant fluid (3900 units) while lower than the whole semen (4900 units), suggests that a considerable amount of LD activity is present in the noncellular fluid component. In 4 patients with azoospermia, LD activity of the semen was 1600, 2400, 4000, and 2400 units respectively, and mean LD activity of 8 semen specimens which were either azoospermic or which contained only an occasional sperm cell was 2700 units. In 20 semen specimens in which the sperm count ranged from 1 to 50 million sperm per cc, mean LD activity was 4400 units. In 24 specimens with sperm counts above 50 million per cc, mean LD activity was 6000 units. In this group, 13 semen specimens with counts above 100 million per cc had mean LD activity of 7800 units. There appears to be a semiqualitative relationship between LD activity of semen and its sperm count, although sperm-free seminal fluid contains significant LD activity. No relationship between motile activity of spermatozoa and LD activity of semen was apparent. From 2 normal individuals, semen specimens showed high LD activity which bore no apparent relationship to semen quality or sperm count; 2 semen specimens obtained 3 days apart from a normal individual had LD activities of 19,000 and 13,000 respectively while specimens from another individual had LD activities of 36,000 and 16,000 respectively.

Comparison of LD activity of the first and second portions of the "split" ejaculate reveals the mean activity to be 13,000 and 4100 units respectively. The first third to one-half of the ejaculate is known to be derived primarily from the prostate and the ampullae of the ductuli deferentia and contains the bulk of the spermatozoa(5). In view of the fact that azoospermic semen contains LD activity, it appears that the prostatic secretions contribute a considerable portion of the total LD activity of human semen.

Although the significance of these observations has yet to be clarified they serve as a baseline for study of the clinical significance of LD alterations of prostatic secretions and of semen in diagnosis of prostatic and testicular malignant neoplasia.

Summary. 1. Lactic dehydrogenase is present in high concentration in normal human semen and in seminal fluid of azoospermic individuals. 2. As compared to normal human blood plasma, LD activity of seminal fluid is high and is comparable to values obtained from tissue fluids which contain or bathe growing malignant cells. 3. There appears to be a semi-qualitative relationship between LD activity of semen and its content of spermatozoa but a considerable portion of LD activity is present in sperm-free seminal fluid. The latter activity seems to be derived principally from the prostatic secretions.

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Essential Role of Hypophysis in Hypercorticism and Hyperovarianism in DBA x CE and Reciprocal Mice.* (24318)

WILLIAM B. ATKINSON† AND MARGARET M. DICKIE
Department of Anatomy, University of Cincinnati College of Medicine, Cincinnati, Ohio, and Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Me.

In a number of inbred strains of mice (DBA (1), CE(2), A, C_{57}BL and C_3H(3)) and in some of their hybrid offspring (C_3H x A(4), DBA x CE(5)) adrenal cortical hyperplasia and/or adenocarcinoma develop in both sexes subsequent to early gonadectomy. Similar adrenal tumors develop in intact NH mice due to spontaneous failure of the gonads at about 9 to 10 months of age(6). That these cortical tumors are endocrinologically active is evidenced by their growth-stimulating effect on the atrophied reproductive organs after they develop in the gonadectomized mice. In some instances the growth induced may become hyperplastic in nature. Hyperplasia of the reproductive tract also occurs in intact DBA x CE and reciprocal hybrid female mice due to spontaneous hyperovarianism that begins to manifest itself at 6 to 7 months of age and which persists throughout the life of the animals(7).

It has been the general consensus of the several authors cited that the hypophysis is involved in both spontaneous and induced adrenal and ovarian endocrinopathies. No direct evidence, however, has been offered in support of this contention. The following experiments were designed to determine whether or not the hypophysis is essential to development of hyperovarianism in intact mice and to development of hypercorticism in neonatally ovariectomized mice.

Procedure. The present study was begun with 82 virgin female DBA x CE and reciprocal hybrid mice, born in the Jackson Laboratory colony and maintained at 70°F and on a diet of Purina laboratory chow and water *ad libitum*. The mice were divided into 4 groups: (1) 24 received no treatment, (2) 8 were ovariectomized within 72 hours after birth, (3) 25 were hypophysectomized at weaning, *i.e.* at approximately 30 days of age,‡ and 25 were ovariectomized shortly after birth and were then hypophysectomized at weaning. The operated animals were weighed occasionally but were subjected to no other treatment.

The mice were sacrificed at from 6 to 9 months of age, the earliest period during which hyperplasia of the ovaries, adrenals and uterus is consistently present in intact animals. Whereas all the operated animals survived the immediate post-surgical period, there was a high delayed mortality rate among the hypophysectomized mice, particularly at from 60 to 90 days of age. Several of the surviving hypophysectomized mice, although much lighter in weight than the intact animals, gained 4 to 5 g and consequently were eliminated from the study because of incomplete removal of the gland. Hence, there were but 5 completely hypophysectomized and 10 hypophysectomized-ovariectomized mice that survived at least to the required age

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† Present address: Dept. of Anatomy, Univ. of Louisville School of Medicine, Louisville, Ky.

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TABLE I. Quantitative Effects of Hypophysectomy on Hypercorticism and Hyperovarianism in DBA × CE and Reciprocal Mice.

Treatment	Age, mo	No. of mice	Body wt, g	Organ wt, mg/100 g body wt			
				Hyp	Adrs	Ovaries	Uterus
None	6-7	12	25.7 ± 3.6*	7.8	21.9	85.3	438.4
	8-9	12	25.9 ± 1.1	7.9	22.1	143.4	558.7
Ovariectomized at birth	8-9	8	34.4 ± 2.0	7.8	27.7	—	99.1
Hypophysectomized at weaning	6-9	5	12.5 ± .5	—	14.9	18.5	40.0
Ovariectomized at birth and hypophysectomized at weaning	6-9	10	13.4 ± .4	—	14.1	—	20.1

* ± S.E.

of 6 months. The animals were autopsied immediately after sacrifice and weights of adrenals, uteri, hypophyses and/or ovaries, if present, were determined to the nearest 0.1 mg on a Roller-Smith torsion balance.

In the neonatally ovariectomized mice there is an increase of approximately 30% in body weight and both an absolute and relative increase in adrenal weight of about the same magnitude as compared with body and adrenal weights in the intact animals of the same age. The uteri in the 8- to 9-month-old castrates do not show the hyperplasia present in the intact mice, but in comparison with the uteri in the hypophysectomized groups, it is evident that in these animals some degree of uterine growth has occurred in conjunction with enlargement of the adrenals. In the hypophysectomized intact and ovariectomized mice, on the other hand, there is little doubt that there is marked failure of ovarian and adrenal growth and absence of uterine stimulation by these organs. The results are summarized in Table I.

Discussion. The present observations on weights of adrenals, ovaries and uteri in 8- to 9-month-old intact and neonatally ovariectomized DBA x CE and reciprocal hybrid mice confirm quantitatively what previously has been described primarily in morphological terms. Whereas the degree of adrenal and uterine enlargement in the 8- to 9-month-old castrates is but a fraction of that which develops after 12 months, selection of this earlier stage in the hypercortical syndrome was necessary due to the difficulty of maintaining the hypophysectomized mice to more advanced ages.

It has been postulated, principally on the basis of theoretical considerations, that adrenal hyperplasia and subsequent cortical tumors that develop in gonadectomized mice of the several strains previously mentioned are the result of stimulation of the adrenals by elevated titers of hypophyseal gonadtropins resulting from removal of the gonads(3). No such formal hypothesis has been offered to explain the hyperovarianism exhibited by intact mice, but the implication of the hypophysis has been assumed explicitly(5,8). The present observations cannot provide a detailed theory of the physiological mechanisms involved in the pathogenesis of the adrenal and ovarian endocrinopathies in these mice, but the data do provide indisputable direct evidence of the essential role of the hypophysis in their development.

Summary. Weights of the adrenals, ovaries, uteri and hypophyses of 6- to 9-month-old DBA x CE and reciprocal hybrid mice were measured in: (1) intact virgins, (2) neonatal castrates, (3) mice hypophysectomized at weaning, and (4) hypophysectomized castrates. There was marked reduction in size of the adrenals, ovaries and uteri in the hypophysectomized intact animals and of adrenals and uteri in the hypophysectomized castrates. Since at 6 to 9 months of age intact mice of these hybrid strains exhibit spontaneous enlargement of ovaries and hyperplasia of the reproductive tract, and animals ovariectomized neonatally exhibit hyperplasia of adrenals and hormonal stimulation of the reproductive tract, the present data provide definitive evidence of the essential role of the hypophysis in these hypercortical and hyperovarian syndromes.

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Effect of Behavior on Development of Resistance in Trichinosis.* (24319)

DAVID E. DAVIS AND CLARK P. READ (Introduced by J. J. Christian)
School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, Md.

In recent years the effect of external conditions on resistance to a pathogen has received increasing attention. This paper describes the effect of behavior upon resistance to *Trichinella spiralis* infections in mice. A series of papers(1,2,3,6) has demonstrated that crowding in mice produces hypertrophy of the adrenal glands and a chain of physiological consequences, especially with respect to reproductive functions. In addition it seemed probable that these adrenal changes would affect resistance. Resistance here is defined as response of a host to past or present experience with a parasitic organism or a chemically related entity, whereas the term insusceptibility is restricted to the properties of a host which render it unsuitable as a habitat for a parasite but which do not represent a response of the host to the parasite(11). This paper presents the first of a series of studies designed to determine alterations of development of resistance in respect to density of the population. The parasite *Trichinella spiralis* was selected because considerable information was available on the mechanisms of resistance and because it was possible to set up an experiment in which transmission from mouse to mouse did not occur(7). It has been shown that during the initial phase of a *Trichinella* infection there is an inflammatory response in the wall of the gut. This response is thought to be involved in determining length of life of the adult worms(9). It has been known for several years that resistance to *Trichinella* is altered by cortisone treatment(10,12). Coker(4,5) showed that when mice are given injections of cortisone the intestinal inflammation in trichinosis is reduced. As a consequence, the life of the adult worms is prolonged and more larvae are presumably deposited in the host's tissues than is the case in hosts not receiving cortisone treatment. Resistance to certain other animal parasites is altered by administration of cortisone to the host(13).

Methods. The general method consisted of infecting mice with *Trichinella,* then arranging part of the mice as a group to contrast the effects of interaction of individuals with the condition of isolation. The mice are called wild strain because they are raised in large cages from mice that are regularly replaced by mice caught in the wild. These mice have been used for a variety of studies of the adrenals(1,6). The young mice are weaned at 20 days and placed in jars separated from each other by partitions so that one mouse cannot see another mouse. Food and water are provided and the mice live in these jars until they are sexually mature at an age of about 4 months. Only male mice are used in these studies.

The grouping procedure consists of placing 6 mice together in a large can for about 4 hours a day. At the end of this time each mouse is put back in his own jar which is kept isolated from other mice. The large can does not have food or water. The mice begin

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TABLE I. Number of Trichinella Found in Isolated and in Grouped Mice.

Exp. I				Exp. II			
Isolated		Grouped		Isolated		Grouped	
Wt of mouse	No. of worms	Wt of mouse	No. of worms	Wt of mouse	No. of larvae	Wt of mouse	No. of larvae
				per g		per g	
28.3	6	32.4	26	28.8	880	30.0	1433
31.5	18	30.2	38	27.7	1043	19.2	1443
27.8	0	28.8	24	25.5	1026	29.2	1543
27.1	0	32.7	29	26.9	1006	26.9	1733
30.1	0	28.5	51	27.5	1093	21.8	1626
27.2	0	24.1	28	27.0	1273		
29.0	3	30.0	40				
28.7	0	27.7	16				
27.2	0	26.5	27				
28.3	0	25.5	50				
25.6	0	24.7	20				

to fight as soon as they are placed in the can and fight vigorously for most of the first 4 hours. During this time the social rank is pretty well settled but some further definition may occur on subsequent days. This grouping is done for 10 days. Generally speaking, while in the can the top-ranking mouse will attack and defeat other mice who in turn may attack lower-ranking individuals. The fighting is severe at first but, by the second or third day, involves only an occasional encounter. The mice spend most of their time sitting in different parts of the can licking themselves or simply resting. Such grouped mice will be referred to as interaction groups.

Control and experimental mice were chosen as follows: The 24 mice were listed according to weight beginning with the heaviest (32.5 g) and ending with the lowest (25.7) and alternately assigned to each group. Thus, the 12 control mice averaged 27.1 g while the mice in the 2 interaction groups averaged 26.6.

Trichinella spiralis was maintained in Princeton Swiss mice and rats of the Sprague-Dawley strain. Worms for experimental infections were obtained by 45 minute peptic digestion of blendorized muscle from the stock animals. The larvae were washed with Ringer's solution by centrifugation and suspended in Ringer's solution containing 0.2% agar at a concentration of 625 larvae per ml. The hosts in all experiments were given a 0.2 ml aliquot of the suspension by stomach tube. The suspension was thoroughly agitated before removal of each aliquot. Mice of the groups were infected in alternative order. Determinations of adult worm burden were made by the methods of Larsh and Kent(8) and larval worm burden by peptic digestion of the weighed muscles and direct counting of aliquot samples. Determinations of worm burden were made by the junior author who did not know the history of the animal in the experiment.

Results. In Exp. I, 24 mice were infected with the standard dose of *T. spiralis* larvae on Day 1. Twelve animals were divided into 2 lots of 6. These were interaction groups during Days 2 through 11, and the remainder were isolated controls. In this experiment, the mice in the interaction groups behaved in the usual manner. Severe fighting persisted to such an extent that the mice were kept together for only 3 hours a day and carefully watched to avoid deaths. During the 10-day period, one experimental and one control mouse died. A social rank in the interaction groups was obvious by the third day; in both groups the second heaviest mouse was dominant. The mice were killed on the fifteenth day after infection, and a determination made of the number of adult *T. spiralis* harbored by each animal. The data obtained are presented in Table I. The adrenals of the 11 control animals averaged 3.92 mg (S.D. = 0.38) while those of the 11 experimentals averaged 4.19 (S.D. = 0.55). This difference is not significant.

In Exp. II the procedures were identical except that the mice were held for a longer

period of time after infection. The animals were infected with *T. spiralis* in Day 1, the interaction group was brought together on Days 2 through 11, and the animals were killed on Day 30. Counts were made of the larvae in the muscles of these animals. The data obtained are presented in Table I. Adrenals of the controls averaged 4.24 mg (S.D. = 0.70) while those of the experimentals averaged 5.08 mg (S.D. = 0.65). This difference is significant (P = 0.01).

Discussion. The results of Exp. I support an hypothesis that the initial resistance response to the adult *Trichinella* has been markedly suppressed. Although histological studies were not made to prove the point, it seems that this is a manifestation of the suppression of intestinal inflammation. The results obtained in Exp. II show that there is an increased burden of larval worms in the fighting animals which is attributed to an alteration of the resistance response. This might well have a dual basis. Since the female worms remain in the intestine for a longer period of time in these animals, it is presumed that more larvae are deposited by the adults. However, we have not ruled out the possibility that suppression of concomitant and subsequent humoral immune responses to the larval worms may also be involved.

Although the mice arranged themselves in a rank the adrenal weights did not increase regularly with decrease in rank. However, as expected the adrenals of the 3 dominant mice were on the average, smaller (4.24 mg) than those of the lower-ranking mice. The number of adult *Trichinella* was not related to rank in any group.

The present experiments have shown that degree of development of resistance to an infectious agent may be in part dependent on socio-psychological factors. These experiments were arranged so that transmission among the mice in the interaction groups was impossible. Food supply was not a limiting factor during the experiments. In some situations the stress of nutritional insufficiency might enhance the effects of increasing social contact. It has been pointed out(11) that dissociation of variables in the field or laboratory analyses of resistance and insusceptibility is difficult and that some of the experimental demonstrations of alterations of immunity in malnutrition may involve adrenal responses rather than direct chemical deficiencies of antibody-synthesizing systems.

It may be noted that these results were obtained with a "wild" strain of mice that have larger adrenals than do domesticated strains. The domesticated strains show the same responses in principle(1) but to a considerably lesser degree. Thus experiments with domesticated mice might not show a detectable response although we have not examined this point.

The possible significance of the present findings to host populations is manifest. Under conditions of increased social contact, the members of a population may be less capable of developing resistance to an infectious agent. In many cases this effect would be compounded by enhanced contagion. The data suggest, though they do not establish, that those individuals that are highest in the social hierarchy would be least affected by the infectious agent. Probable effects in the evolution of vertebrate populations are obvious. The alteration of host resistance would also have implications for parasite populations. Suppressed host resistance responses might allow the exploitation of new host species by some parasitic species, with subsequent selection of individuals adapted for life in the new host.

Summary. The demonstration that behavioral factors cause hypertrophy of adrenals, and that injection of corticoids decreased the resistance of mice to *Trichinella spiralis* suggested that behavior might affect infections under relatively natural conditions. "Wild" strain house mice were infected with embryonated larvae. Twelve mice were isolated in jars and 12 were placed in 2 groups in a large can for 3 hours a day for 10 days. When killed on the fifteenth day the grouped mice had from 16-51 worms in the intestine while only 3 of the isolated mice had worms. To determine the effect on subsequent stages, 12 mice were infected. Six were isolated and 6 were grouped for 10 days. All were killed on the thirtieth day. The isolated mice averaged 1054 larvae per gram of muscle while the

grouped mice averaged 1556. It is concluded that behavior may affect resistance and it is suggested that this phenomenon may be an important factor in epidemics and in development of host-parasite relations.

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Enzyme Studies in Muscular Dystrophy. III. In Hereditary Muscular Dystrophy in Mice.*† (24320)

I. M. WEINSTOCK,‡ SANDRA EPSTEIN AND A. T. MILHORAT
Depart. of Medicine, Cornell University Medical College, Russell Sage Institute of Pathology, and N. Y. Hospital, N. Y. City

Michelson, Russell and Harman(1) reported on spontaneous occurrence of a genetic mutation resulting in muscular dystrophy in a highly inbred strain of mice. The muscular areas involved, the nature of the lesions in these muscles, and lack of involvement of the nervous system, are similar to those seen in muscular dystrophy in man. By these criteria, this form of muscular dystrophy in the mouse would appear to be related more closely to muscular dystrophy in patients than are the experimentally induced types of muscle wasting. To characterize further this myopathy in mice, and if possible, to find some relationship to other forms of muscle wasting, we have investigated a number of enzyme systems in the muscles. Our results will be discussed in relation to those of comparable studies in muscular wasting of Vit. E-deficiency and of neurotomy, and that occurring in clinical forms of muscular dystrophy in man.

Methods and materials. Mice of strain 129 (dystrophic animals and normal litter mates) obtained from the Roscoe B. Jackson Memorial Laboratory were given a commercial mouse pellet diet. (Purina Laboratory Chow). The animals were allowed free access to food and water. According to the original investigators(1), symptoms of muscular dystrophy are evident by the third week of life, and dystrophic mice survive from one to 6 months, or occasionally longer. In these investigations, age of the dystrophic mice averaged 68 days (46-110), and weight averaged 14.1 g (9.7-19.0), when they were sacrificed. The control group consisting of heterozygous litter mates of the dystrophic mice, averaged 63 days (47-116) of age, and 21.4 g (16.4-25.4) in weight when they were studied. Dystrophic animals were smaller in size and lighter in weight than controls, and exhibited various degrees of ataxia, atrophy, and paralysis. However, there was no close correlation between age or weight of the animals, within the ranges observed, and gross symptoms of muscular dystrophy.

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‡ Present address: Dept. of Physical Medicine and Rehabilitation, N. Y. Medical College, and Bird S. Coler Hospital, N. Y. City.

TABLE I. Effect of Muscular Dystrophy on Composition of Mouse Muscle.

Types and No. of animals	Content/g wet wt*				$\frac{CN}{TN}$§ × 100, %
	Total N_2	Protein† N_2	Collagen‡ N_2	Dry wt	
	mg				
Controls (14)	20.3 ± 2.3‖	18.9 ± 2.0	1.5 ± .5	165 ± 21	7.4
Dystrophic (12)	17.3 ± 1.9	16.3 ± 1.8	1.9 ± .2	156 ± 16	11.0
% change	−15	−14	+27	−5.5	
P¶	<.01	<.01	<.02	<.3	

* Tissues were kept saturated with water at 0°C, while visible fat and connective tissue were removed. Small pieces and strips of muscle tissue were blotted before weighing, but appreciable water still adhered to tissue surfaces. Dry wt determinations indicate reproducibility of technic.

† Includes collagen nitrogen.

‡ Collagen was determined directly by analysis of alkali insoluble residue.

§ $\frac{CN}{TN} = \frac{\text{Collagen nitrogen}}{\text{Total nitrogen}}$. ‖ Stand. dev. ¶ P = Level of significance.

The mice were sacrificed by decapitation. Muscle from the entire hind quarters, after removal of visible fat and connective tissue, was minced, then homogenized in water in a glass Potter-Elvehjem apparatus. Aliquots of the homogenate were diluted with water and used for assays of enzyme activity. Samples of the homogenate were dried in an oven at 105-110°C for 18-20 hours for dry weight determinations. Collagen was determined by a slight modification of the method of Lilienthal *et al.*(2), and nitrogen was estimated by direct nesslerization after digestion.

Aldolase was determined in the system of Sibley and Lehninger(3), with some modifications as suggested by Dounce *et al.*(4). Triose phosphate was measured as the difference in amounts of inorganic phosphate, before and after alkaline hydrolysis(5). Inorganic phosphate was determined by the Lowery-Lopez method(6) modified by Potter(7). Two different levels of enzyme concentration, in duplicate, were used in each determination of aldolase activity, and the results were averaged. Succinoxidase and cytochrome oxidase activities were determined by the methods of Schneider and Potter(8,9). Oxygen consumption was linear for at least 30-40 minutes, and average values of the linear portion of the curve were used to calculate oxygen consumption per hour. Cytochrome oxidase activity was determined at 3 levels of enzyme concentration, and the results were extrapolated and corrected for zero enzyme concentration(9). Cathepsin activity was determined by a modified Anson procedure(10) previously described(11). The substrate was hemoglobin at pH 4.0, and proteolysis, in the absence of added activators, was measured by increase in optical density at 280 mμ.

Results. Because of the changes in composition of muscle associated with most wasting conditions, a proper reference base for enzyme activity is critical. Concomitantly with these enzyme studies, we determined dry weight, and total-, protein-, and collagen-nitrogen of the muscle. Results of the enzyme assays are reported on the basis of wet weight, dry weight, protein-nitrogen content and non-collagen protein nitrogen content of the muscle. Probably the most accurate index of functioning muscle mass upon which to base enzyme activity is non-collagen protein nitrogen content(2,12). An apparent decrease in enzyme activity, unless based on content of non-collagen protein, might be nonspecific and only a reflection of over-all breakdown of muscle. The effects of muscular dystrophy on composition of the muscle of these mice are shown in Table I. There was a significant 15% decrease in total nitrogen and protein nitrogen content of the muscle when estimated on a wet weight basis, although dry weight of the muscle was unchanged. Collagen nitrogen increased as much as 27%, but accounted for only 11% of the total nitrogen because of the relatively small amounts present initially.

Data on aldolase activity are shown in Table II. In dystrophic muscle, aldolase activity is decreased when estimated on the basis of either wet or dry weight of muscle. How-

TABLE II. Effect of Muscular Dystrophy on Aldolase Activity of Mouse Muscle.

Types and No. of animals	μmoles fructose-diphosphate split/hr			
	Per g wet wt	Per mg dry wt	Per mg PN*	Per mg NCPN†
Controls (12)	1390 ± 350	8.4 ± 1.6	72 ± 14	79 ± 16
Dystrophic (11)	1090 ± 330	7.0 ± 1.9	68 ± 23	76 ± 25
% change	−22	−17	−6	−4
P	<.05	<.05	<.6	<.7

* PN = Protein nitrogen. † NCPN = Non-collagen protein nitrogen.

ever, when based on content of either protein nitrogen or non-collagen protein nitrogen in the muscle, aldolase activity in dystrophic muscle is not significantly different from that in the normal controls. Succinoxidase and cytochrome oxidase activities of the muscles are shown in Table III. There was no significant difference between succinoxidase activities of control and dystrophic groups, regardless of which reference base was used for estimating enzyme activity. On the other hand, cytochrome oxidase activity was considerably increased. When referred to either wet or dry weight of the muscle, it was increased about 35%, and when this activity was based on non-collagen protein nitrogen content of the muscle, the increase was about 50%, since non-collagen protein nitrogen content of the muscle decreased during the course of dystrophy. Cathepsin activity of muscle of dystrophic mice was increased over the levels of the litter mate controls (Table IV). This increase in cathepsin activity was significant when based on any of the employed standards of muscle mass, and, as in the case of cytochrome oxidase activity, the increase was greatest when based on non-collagen protein nitrogen content of the muscle.

Although the activities of both cytochrome oxidase and cathepsin in dystrophic muscle were elevated, there was no obvious correlation between these changes, and age or weight of the dystrophic mice, within the ranges used in this investigation. However, there did seem to be some general relation between increases in enzyme activity and gross symptoms of muscular dystrophy. Cytochrome oxidase and cathepsin activities in the muscles of 2 homozygous normal control mice were of the same order as those in the heterozygous normal control group. Concurrently, with the studies reported here, we similarly assayed and found no change in the activities of these enzymes in liver tissue.

Discussion. The changes in protein content of muscles of the dystrophic mouse observed (Table I) were similar to those reported in 3 other types of muscle wasting, *i.e.*, wasting that occurs after Vit. E-deficiency (13,14), atrophy that results from neurotomy (12), and degeneration of muscles that represents the main pathologic alteration in muscular dystrophy in patients(14,15). Although in these forms of muscle wasting, increase in collagen content of the muscle is quantitatively greater, the general result of all forms of wasting is a reduction in non-collagen protein nitrogen content of the muscle.

Since aldolase activity of dystrophic muscle appears decreased only when based on wet or

TABLE III. Effect of Muscular Dystrophy on Succinoxidase and Cytochrome Oxidase Activities of Mouse Muscle.

Types and No. of animals	Succinoxidase activity, mm^3 O_2/hr				Cytochrome oxidase activity, mm^3 O_2/hr			
	Per g wet wt	Per mg dry wt	Per mg PN	Per mg NCPN	Per g wet wt	Per mg dry wt	Per mg PN	Per mg NCPN
Controls (9)	7400 ± 1200	45 ± 9	380 ± 79	424 ± 84	18,300 ± 4200	116 ± 35	990 ± 260	1080 ± 290
Dystrophic (7)	7100 ± 1100	46 ± 9	415 ± 81	470 ± 92	24,900 ± 3700	159 ± 20	1460 ± 250	1660 ± 280
% change	−4	+2	+5	+11	+36	+37	+47	+54
P	<.6	<.8	<.3	<.2	<.01	<.01	<.01	<.01

TABLE IV. Effect of Muscular Dystrophy on Cathepsin Activity of Mouse Muscle.

Types and No. of animals	Cathepsin activity*			
	Per g wet wt	Per mg dry wt	Per mg PN	Per mg NCPN
Controls (8)	10.7 ± 2.7	.066 ± .017	.58 ± .13	.62 ± .14
Dystrophic (6)	17.7 ± 4.6	.110 ± .037	1.02 ± .26	1.16 ± .31
% change	+65	+67	+76	+87
P	<.01	<.01	<.01	<.01

* Cathepsin activity = Change in optical density at 280 mμ/ml of incubation mixture/hr.

dry weight of muscle, and is of normal value when estimated on the protein- or non-collagen protein nitrogen, it is probable that any changes noted are only a reflection of over-all reduction in functioning muscle mass. Serum aldolase of dystrophic mice has been found to be increased(16). A similar relationship, between blood serum and muscle enzyme activities, has been reported for transaminase in patients with muscular dystrophy; *i.e.*, serum transaminase activity was increased, while muscle transaminase levels, when referred to non-collagen protein nitrogen, were unchanged (17). Increase in activity of an enzyme in blood serum does not necessarily indicate that the metabolic lesion specifically involves that particular enzyme, since the increase may be only a reflection of tissue destruction. Phosphorylase activity of muscle of dystrophic mice has been investigated by Leonard(18), who found approximately a 30% decrease in activity of dystrophic thigh muscle, when based on wet weight of tissue. Data on non-collagen protein nitrogen content of the muscles were not reported. Reduction in activity of both aldolase and phosphorylase in the muscle of the dystrophic mouse is much less than has been observed in patients with muscular dystrophy. The muscles of these patients may show reductions in aldolase and phosphorylase activities to one-third their normal values, when based on non-collagen protein nitrogen content of the muscle(17).

Although succinoxidase activity of dystrophic mouse muscle tends to show a slight increase on the basis of non-collagen nitrogen content, this difference is not significant, and one must conclude that succinoxidase activity is essentially unchanged. On the other hand, cytochrome oxidase activity is significantly increased in the muscle of the dystrophic mouse. However, none of the respiratory or glycolytic enzymes investigated in this laboratory (succinoxidase, cytochrome oxidase, and aldolase), or other representative respiratory or glycolytic enzymes observed by others, show a pattern of change that is similar in the 4 types of muscle wasting mentioned here. Alterations in activities of respiratory and glycolytic enzyme systems of muscle, associated with various muscle wasting conditions, have been reported(12-15,17,19,20). A possible, though tentative conclusion, that may be drawn from available evidence, is: in clinical muscular dystrophy and in muscle atrophy due to neurotomy, glycolytic enzyme systems are generally decreased, and respiratory enzymes are not primarily affected; in muscular dystrophy resulting from Vit. E-deficiency, and in the hereditary muscle dystrophy in mice, respiratory activity tends to be increased, and glycolytic enzymes are relatively unchanged. This conclusion is based on experimental observations that often are not definitive, and, occasionally are conflicting. It is perhaps equally likely that the changes observed in enzyme activity are a reflection of specifically different causative mechanisms of muscle wasting, or are merely secondary effects of the atrophy.

However, in all of the 3 forms of muscle wasting in which it has been investigated, cathepsin activity has been found to be increased. Moreover, of all the enzyme systems investigated, cathepsin alone shows a common pattern of change. In the present studies, cathepsin activity was increased nearly 2-fold in dystrophic mouse muscle. Similar increments have been observed in the muscle of rabbits with muscular dystrophy due to Vit. E-deficiency(11), and in muscle following neurotomy(21,22). Although the role of the catheptic enzymes, *in vivo*, has not yet been clearly established, they may be in-

volved in protein catabolism, and may be the common mechanism in muscle wasting due to a number of different causes. It is of interest that cathepsin activity, *in vitro,* has been reported to correlate with gross changes in protein metabolism and mass in regressing tumors(23), and with certain hormonal influences(24). Investigation of this enzyme system in the muscles of patients with muscular dystrophy is planned.

Summary. Changes in muscle composition, and activity of 4 enzyme systems in mice with *dystrophia muscularis,* have been investigated. Total nitrogen and protein nitrogen contents of dystrophic muscle were reduced as compared to controls, while collagen nitrogen was increased. Percentage dry weight was unchanged. Aldolase activity of dystrophic muscle was decreased on the basis of wet or dry weight, but was unchanged on the basis of protein- or non-collagen protein nitrogen content of muscle. Succinoxidase activity was not significantly affected by muscle dystrophy. Cytochrome oxidase and cathepsin activities were markedly increased in the muscle of dystrophic mice as compared to controls. Liver tissue from these mice showed no change as a result of the dystrophy. These results are compared to those observed in other forms of muscle wasting.

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